From Caloric Restriction to Cardiovascular Health: A Protective Role for Sirt3 and Sirt6 in Atherothrombosis

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Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

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Zürich, 2017

Acknowledgements

I would like to thank the Foundation for Cardiovascular Research in Zurich, the Swiss National Science Foundation, the University Research Priority Program Integrative Human Physiology at the University of Zurich, the Hartmann-Müller Foundation, the University of Zurich, the National Institute of Health (USA), SystemsX, the European Research Council, the École Polytechnique Fédérale de Lausanne, and the Swiss Heart Foundation in Bern for funding the projects presented in this dissertation.

Furthermore, I would like to thank my supervisor, Christian Matter, for providing me with this interesting research topic, for his continuous support throughout my time in Zurich, for always respecting my opinion, and for giving me the freedom to follow my ideas, which helped me a lot to become an independent researcher.

I would also like to thank the members of my PhD committee, Michael Hottiger, Ian Frew, and Michael Potente, for their interest in my project, their support, and their scientific input in the annual PhD committee meetings.

Moreover I would like to thank Thomas Lüscher for giving me the chance to work in the Center for Molecular Cardiology, for providing the lab and infrastructure, and for his input at the lab meetings.

I am grateful to all my colleagues at the CMC for their support in the conduction of experimental procedures, many discussions, which helped me to advance my projects, but also for some well-needed distraction in stressful times. I would especially like to thank Julien Weber for helping me wherever he could and for being a good friend in and outside the lab, Stephan Winnik for his guidance in the beginning of my PhD, Lambertus vanTits for his support and great help in writing grants, and Natacha Calatayud and Lisa Pasterk for their experimental input as 'my students' and many funny hours.

I would like to thank Natacha Calatayud, Jürgen Pahla and Julien Weber for carefully proofreading this dissertation.

Last but not least, I would like to thank my family and friends for their support and encouragement in the past 4.5 years. I especially would like to thank AK for moving to Zurich, keeping up with my lab-induced moods, and making my time in Switzerland even more enjoyable.

Table of contents

1 Abstract

Background: Cardiovascular disease (CVD) represents a major health burden and is the world's leading cause of mortality. The most common pathological conditions in CVD are endothelial dysfunction and atherosclerosis. The most frequent complication is arterial thrombosis, which may lead to myocardial infarction and stroke. Atherogenesis is characterised by the occurrence of chronic inflammatory processes and involvement of reactive oxygen species (ROS). Recently, ROS-mediated formation of neutrophil extracellular traps (NETs) was associated with atherothrombosis and subsequent major adverse cardiovascular events.
Sirtuins are a family of seven NAD^+-dependent protein deacetylases that play a beneficial role in metabolism and age-related processes and are activated upon caloric restriction. Sirtuin 3 (Sirt3) is located in mitochondria, where it governs mitochondrial metabolism. Mitochondria are major producers of ROS and Sirt3 protects the cell from ROS by activating superoxide dismutase 2 (SOD2) and by increasing transcription of SOD2 and Catalase, the main mitochondrial ROS scavengers. Sirtuin 6 (Sirt6) is located in the nucleus, where it regulates inflammation, DNA maintenance, and glucose and lipid metabolism. Sirt6 inhibits inflammation by interacting with subunits of nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) and subsequently deacetylating lysine 9 of Histone 3 (H3K9) to attenuate NF-κB- and AP-1-mediated transcription of pro-inflammatory genes. Current studies suggest that this mechanism may also occur in endothelial cells. NF-κB and AP-1 also regulate the expression of tissue factor (TF), a central initiator of blood coagulation.
Of note, the functions of Sirt3 in atherosclerosis and endothelial function, as well as the roles of Sirt3 and Sirt6 in arterial thrombosis, have not yet been investigated.

Methods: Sirt3-deficient ($Sirt3^{-/-}$) mice were used to investigate the causal role of Sirt3 in vascular disease. For assessing atherosclerosis, $Sirt3^{-/-}$ mice were crossbred with low-density lipoprotein receptor (LDL-R) depleted mice and 8-week-old males were fed a 1.25% (w/w) high-cholesterol diet for 12 weeks to induce atherosclerosis. Atherosclerosis was evaluated in thoracoabdominal aortae *en face* and in cross sections of aortic roots. In addition, metabolic rate and systemic oxidative stress were assessed using indirect calorimetry and quantification of the oxidative stress marker malondialdehyde.
To induce endothelial dysfunction, 8-week-old $Sirt3^{-/-}$ mice were fed a 1.25% (w/w) high-cholesterol diet for 12 weeks. Subsequently, aortic rings were isolated and endothelium-dependent relaxation assessed in an organ chamber bath. Molecular effects of Sirt3 deficiency were analysed using a siRNA-mediated knockdown of Sirt3 in cultured human aortic endothelial cells (HAECs).
For thrombosis experiments, 16-week-old $Sirt3^{-/-}$ mice were stimulated by an intraperitoneal injection of 5 mg/kg lipopolysaccharide (LPS). To investigate *in vivo* time to thrombotic occlusion, mice were subjected to laser-induced *in vivo* carotid thrombosis. To examine *ex vivo* clotting properties, blood was analysed using rotational thromboelastometry (ROTEM). Moreover, neutrophils were isolated from bone marrow and stimulated with LPS to assess formation of NETs.

$CD14^+$ leukocytes from patients suffering from acute ST-elevation myocardial infarction (STEMI) were analysed for transcription levels of Sirt3 and SOD2.
Finally, endothelium-specific Sirt6 deletion in mice was generated using the VE-cadherin promoter and carotid thrombosis was induced as described above to investigate the effects of endothelial Sirt6 loss-of-function in thrombosis. To study the molecular effects of Sirt6 deficiency on endothelial cells, Sirt6 knockdown was performed in cultured HAECs.

Results: Absence of Sirt3 did not affect atherosclerosis but increased systemic oxidative stress, accelerated weight gain and impaired adaptation to rapid changes in nutrient supply.
Loss of Sirt3 caused mild endothelial dysfunction and increased oxidative stress in endothelial cells. Sirt3-deficient HAECs were protected from ROS-induced cell death via a C/EBP-β-dependent rescue mechanism that induced expression of SOD2.
Time to thrombotic carotid occlusion was cut in half in $Sirt3^{-/-}$ mice. Clot formation was accelerated and clot stability increased compared to controls. Furthermore, increased levels of active soluble TF were measured in the blood of $Sirt3^{-/-}$ mice. In neutrophils, Sirt3 deletion decreased SOD2 transcription and increased NET formation. In parallel, leukocytes of STEMI patients exhibited reduced transcription of Sirt3 and SOD2.
Specific deletion of Sirt6 in mouse endothelium decreased time to carotid thrombotic occlusion by 45%. In line with these *in vivo* findings, knockdown of Sirt6 in HAECs increased transcription of pro-inflammatory targets of NF-κB and AP-1 as well as amount and activity of TF.

Conclusions: Deletion of Sirt3 increases systemic and cellular ROS levels, and soluble TF levels in the blood, and thus favours development of cardiovascular metabolic risk factors, endothelial dysfunction, and arterial thrombosis.
Endothelium-specific deletion of Sirt6 accelerates arterial thrombosis by an increase in pro-inflammatory signalling, increased TF presence and activity in endothelial cells.
These results suggest that endogenous Sirt3 and Sirt6 bear the potential to aid in the prevention of endothelial dysfunction, and arterial thrombosis and make them interesting targets for future therapeutic testing.

2 Zusammenfassung

Hintergrund: Kardiovaskuläre Krankheiten (KVK) stellen eine massive Gesundheitsbelastung dar und sind weltweit die Haupttodesursache. Die am häufigsten auftretenden Krankheitserscheinungen bei KVK sind endotheliale Dysfunktion und Arteriosklerose. Die häufigste Komplikation ist arterielle Thrombose, die zu einem Herzinfarkt oder Schlaganfall führen kann. Die Bildung von Arteriosklerose wird typischerweise von Entzündungsreaktionen und der Formation von reaktiven Sauerstoffspezies (RSS) begleitet. Unlängst wurde die Bildung sogenannter *neutrophil extracellular traps* (*deutsch*: neutrophile extrazelluläre Fallen, NETs) in Zusammenhang mit KVK und ihren negativen Folgeerscheinungen gebracht.
Die Sirtuine sind eine Proteinfamilie bestehend aus sieben NAD^+-abhängigen Deacetylasen. Sie werden durch Kalorienrestriktion aktiviert und haben wichtige vorteilhafte Funktionen im Stoffwechsel und bei altersbedingten Krankheiten. Sirtuin 3 (Sirt3) befindet sich in den Mitochondrien, wo es den dortigen Stoffwechsel steuert. Mitochondrien sind Haupterzeuger von RSS und Sirt3 beschützt die Zelle vor diesen reaktiven Substanzen in dem es Superoxiddismutase 2 (SOD2) aktiviert und die Transkription von SOD2 und Catalase, den zwei wichtigsten RSS-Inaktivatoren in Mitochondrien, fördert. Sirtuin 6 (Sirt6) befindet sich im Zellkern und reguliert dort Entzündungsreaktionen, Reparatur von DNS-Schäden, sowie Glukose- und Fettmetabolismus. Sirt6 hemmt Entzündungen in dem es mit Untereinheiten von Nuklearem Faktor kappa B (NF-κB) und Aktivatorprotein 1 (AP-1) interagiert und anschliessend Lysin 9 an Histon 3 (H3K9) deacetyliert, was die NF-κB und AP-1 bedingte Transkription entzündungsfördernder Gene bremst. Aktuelle Studien suggerieren, dass dieser Mechanismus auch in Endothelzellen abläuft. NF-κB und AP-1 regulieren ausserdem die Expression von *Tissue Factor* (*deutsch*: Gewebefaktor; TF), einem Hauptinitiator der Blutkoagulation.
Die Bedeutung von Sirt3 in Arteriosklerose und Endotheldysfunktion sowie die Rolle von Sirt3 und Sirt6 in arterieller Thrombose sind bisher nicht bekannt.

Methoden: Die Rolle von Sirt3 in KVK wurde in Mäusen mit einem Sirt3-Defizit ($Sirt3^{-/-}$) untersucht. Um einen Effekt auf Arteriosklerose evaluieren zu können, wurden $Sirt3^{-/-}$-Mäuse mit zusätzlicher Deletion des low-density lipoprotein (Lipoprotein mit niedriger Dichte) Rezeptors (LDL-R) gezüchtet und ab einem Alter von 8 Wochen für 12 Wochen mit einer cholesterinreichen Nahrung (1.25% Cholesterin (w/w)) gefüttert, um Arteriosklerose zu verursachen. Arteriosklerose wurde in thorakoabdominellen Aortae *en face* und in Schnitten von Aortenwurzeln beurteilt. Zusätzlich wurde die Stoffwechselrate und systemischer oxidativer Stress mittels indirekter Kalorimetrie und Quantifizierung des oxidativen Stressmarkers Malondialdehyd gemessen.
Weiterhin wurden 8 Wochen alte $Sirt3^{-/-}$-Mäuse für 12 Wochen mit einer cholesterinreichen Nahrung gefüttert um die Endothelfunktion zu beeinträchtigen. Aortenringe wurden isoliert und endothelabhängige Relaxation in einem Organbad getestet. Um molekulare Auswirkungen eines Sirt3-Verlustes zu untersuchen, wurde in menschlichen Aortaendothelzellen (MAEZs) mittels kleiner RNS-Fragmente die Sirt3-Proteinexpression gehemmt.

Zur Untersuchung von arterieller Thrombose wurden 16 Wochen alte Sirt3$^{-/-}$-Mäuse intraperitoneal mit 5 mg/kg Lipopolysaccharid (LPS) injiziert. In diesen Mäusen wurde daraufhin mittels Laserbestrahlung Thrombose in der rechten Karotis ausgelöst, und die Zeit bis zum thrombotischen Verschluss *in vivo* gemessen. Das Blut der Sirt3$^{-/-}$-Mäuse wurde mittels Thromboelastometrie (ROTEM) *ex vivo* auf seine Gerinnungseigenschaften untersucht. Zusätzlich wurden Neutrophile aus dem Knochenmark der Mäuse isoliert und mit LPS zur Bildung von NETs angeregt. CD14^{+}-Leukozyten aus Patienten, die an einem akuten Myokardinfarkt mit ST-Hebung (STEMI) litten wurden auf Transkription von Sirt3 und SOD2 getestet.
Abschliessend wurde unter Verwendung des vaskulären endothelialen Cadherinpromotors eine endothelzellspezifische Deletion von Sirt6 in Mäusen generiert, in denen wie beschrieben Thrombose ausgelöst wurde, um die Rolle von endothelialem Sirt6 in dieser Erkrankung zu studieren. Um Effekte auf molekularer Ebene zu untersuchen wurde die Sirt6-Expression in HAECs unterdrückt.

Ergebnisse: Arteriosklerose war in Abwesenheit von Sirt3 nicht verändert. In Sirt3$^{-/-}$-Mäusen konnten allerdings erhöhte Werte systemischen oxidativen Stresses, beschleunigte Gewichtszunahme und schlechte Anpassung an Veränderungen im Nahrungsangebot festgestellt werden.
In Endothelzellen verursachte der Sirt3-Verlust erhöhten oxidativen Stress, und schwache endotheliale Dysfunktion. Ein C/EBP-β-abhängiger Mechanismus, der SOD2-Expression induzierte, schützte HAECs vor Zelltod durch erhöhte RSS.
Die thrombotische Verschlusszeit in Sirt3$^{-/-}$-Mäusen verkürzte sich im Vergleich zu Kontrolltieren um die Hälfte einhergehend mit einer beschleunigten Gerinnselbildung und erhöhter Gerinnselstabilität. Ausserdem konnten erhöhte TF-Spiegel im Blut der Sirt3$^{-/-}$-Mäuse gemessen werden. Deletion von Sirt3 in Neutrophilen verringerte die Transkription von SOD2 und erhöhte die Bildung von NETs. Parallel dazu zeigten Leukozyten von STEMI-Patienten eine verringerte Gentranskription von Sirt3 und SOD2.
Fehlendes Sirt6 in Endothelzellen verringerte die Zeit bis zum thrombotischen Verschluss der Karotis in Mäusen um 45%. Entsprechend dieser Beobachtungen *in vivo* zeigte sich in HAECs mit verringerter Sirt6-Expression eine erhöhte Menge und Aktivität von TF, sowie eine Aktivierung NF-κB und AP-1 regulierter, entzündungsfördernder Gene.

Schlussfolgerungen: Deletion von Sirt3 erhöht systemische sowie zelluläre RSS und TF-Spiegel im Blut und fördert daher die Entwicklung kardiovaskulärer Risikofaktoren, endotheliale Dysfunktion und arterielle Thrombose.
Endothelzellspezifisches Fehlen von Sirt6 beschleunigt arterielle Thrombose durch Entzündungsreaktionen und erhöht Präsenz und Aktivität von TF in Endothelzellen.
Die Resultate suggerieren, dass endogenes Sirt3 und Sirt6 das Potential in sich tragen in der Prävention und akuten Versorgung endothelialer Dysfunktion und arterieller Thrombose unterstützend zu wirken. Dies macht sie zu interessanten Kandidaten für zukünftige therapeutische Tests.

3 List of abbreviations

129	Inbred mouse strain 129
AceCS2	Acetyl coenzyme A-synthase
ACh	Acetylcholine
ACS	Acute coronary syndromes
ADP	Adenosine diphosphate
AP-1	Transcription factor activator protein 1
ATP	Adenosine triphosphate
BMI	Body mass index
c-JUN	AP-1 transcription factor subunit
c-MYC	Cellular homologue to viral myelocytomatosis oncogene
C57BL/6	Inbred mouse strain C57 black 6
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CCR2	C-C chemokine receptor type 2
CD14	Cluster of differentiation 14
cGMP	Cyclic guanosine monophosphate
CoA	Coenzyme A
COX	Cyclooxygenase
CtIP	C-terminal binding protein interacting protein
CVD	Cardiovascular disease
CXCR2	C-X-C motif chemokine receptor 2
DNA	Deoxyribonucleic acid
DSB	DNA double-strand break
E-selecting	Endothelial selectin
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FX	Coagulation factor X
FXa	Activated coagulation factor X
G3BP	Ras-GAP SH3 domain binding protein
GCN5	General control non-repressed protein 5
GDH	Glutamate dehydrogenase
H3	Histone 3
H_2O_2	Hydrogen peroxide
HIF1α	Hypoxia-inducible factor 1-alpha
I-TAC	Interferon-inducible T-cell alpha chemoattractant
ICAM-1	Intercellular adhesion molecule 1

IDH2	Isocitrate dehydrogenase 2
IL	Interleukin
IFN-γ	Interferon gamma
IP-10	Inducible protein 10
K	Lysine
LCAD	Long-chain acyl CoA dehydrogenase
LDL	Low-density lipoprotein
LDL-R	Low-density lipoprotein receptor
LPS	Lipopolysaccharide
M-CSF	Macrophage-stimulating factor
MCP-1	Monocyte chemoattractant protein 1
Mig	Monokine induced by gamma interferon
MMP	Matrix metalloproteinase
NET	Neutrophil extracellular trap
NO	Nitric oxide
NAD^+	Oxidised form of nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenosine dinucleotide phosphate
NF-κB	Nuclear factor kappa B
NFATc2	Nuclear factor of activated T-cells 2
NOS	Nitric oxide synthase
O_2^-	Superoxide
$ONOO^-$	Peroxynitrite
oxLDL	Oxidized low-density lipoprotein
P_i	Inorganic phosphate
P-selectin	Platelet selectin
PARP1	Poly [ADP-ribose] polymerase 1
PCSK9	Proprotein convertase subtilisin/kexin type 9
RNA	Ribonucleic acid
ROS	Reactive oxygen species
ROTEM	Rotational thromboelastometry
RT-PCR	Real-time quantitative polymerase chain reaction
SDH	Succinate dehydrogenase
Sirtuin	Silent information regulator 2 protein
Sirt3	Sirtuin 3
Sirt6	Sirtuin 6
SMC	Smooth muscle cell
SNF2H	Sucrose nonfermenting 2 homologue (SMARCA5)

SOD2	Endothelial superoxide dismutase (MnSOD)
SREBP	Sterol-regulatory element binding protein
STAT3	Signal transducer and activator of transcription 3
STEMI	ST-elevation myocardial infarction
SYTOX	SYTOX green nucleic acid stain
TCA	Tricarboxylic acid
TF	Tissue Factor (FIII; CD142; Thromboplastin)
TFPI	Tissue factor pathway inhibitor
Th cell	T helper cell
Tie2	TEK receptor tyrosine kinase (Angiopoietin-1 receptor)
TNF-α	Tumour necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule 1
VE-Cadh	Vascular endothelial cadherin (Cadherin-5)
VSMC	Vascular smooth muscle cell
vWF	Von Willebrand factor
WB	Western Blot
WT	Wildtype

4 Introduction

4.1 Relevance of cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of death worldwide (***Figure 1***).[1] Between 2005 and 2015, the number of global CVD deaths increased by 12.5% to a total number of 17.9 million deaths in 2015. More than 85% of these deaths were caused by ischemic heart disease and stroke.[2] Interestingly, an age-standardisation of cardiovascular deaths in the past decade shows, that the global burden of cardiovascular mortality in relation to the total world population decreased by 15.6%.[2] This may mainly be due to the fact that cardiovascular care is improving, but cannot keep pace with the growth of the global population.[3,4] At the same time, however, many risk factors for CVD are increasing, particularly obesity, diabetes mellitus, and age.[5-7] Thus, in the future, the prevalence of CVD is likely to rise again and the costs for treatment will grow substantially.[8,9] This emphasises the urgent need for novel strategies to effectively prevent CVD.

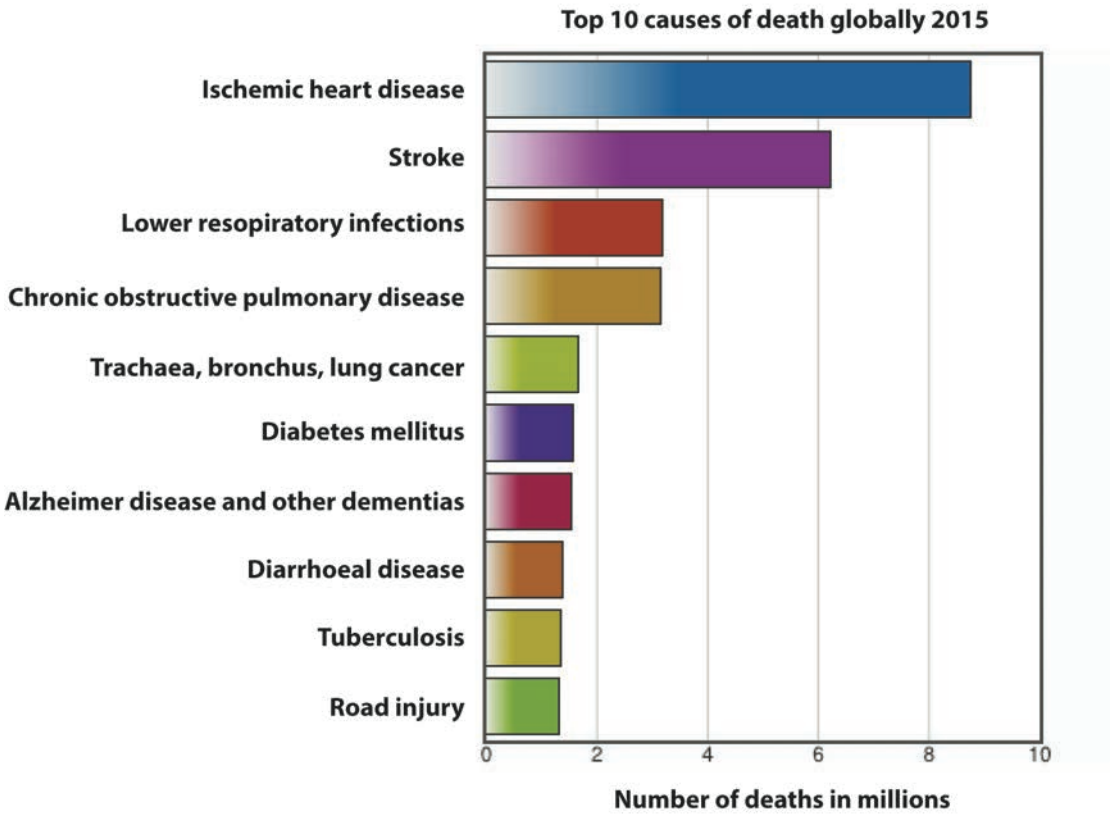

Figure 1. Top 10 causes of death globally 2015. *Source: World Health Organisation (http://www.who.int/mediacentre/factsheets/fs310/en/).*

4.2 The endothelium

Arterial wall

The normal arterial vessel wall consists of three layers (***Figure 2***). The outermost layer of the wall, the tunica externa (externa), mainly consists of connective tissue like collagen, which stabilizes and anchors the vessel to surrounding organs. The middle layer, the tunica media (media), is mainly a smooth muscle cell (SMC) layer and is separated from the externa by the external elastic membrane. Contraction of the SMCs regulates vessel diameter, determines regional blood flow, and systemic blood pressure. The innermost layer of an artery, the tunica intima (intima), consists of endothelial cells supported by the internal elastic membrane.

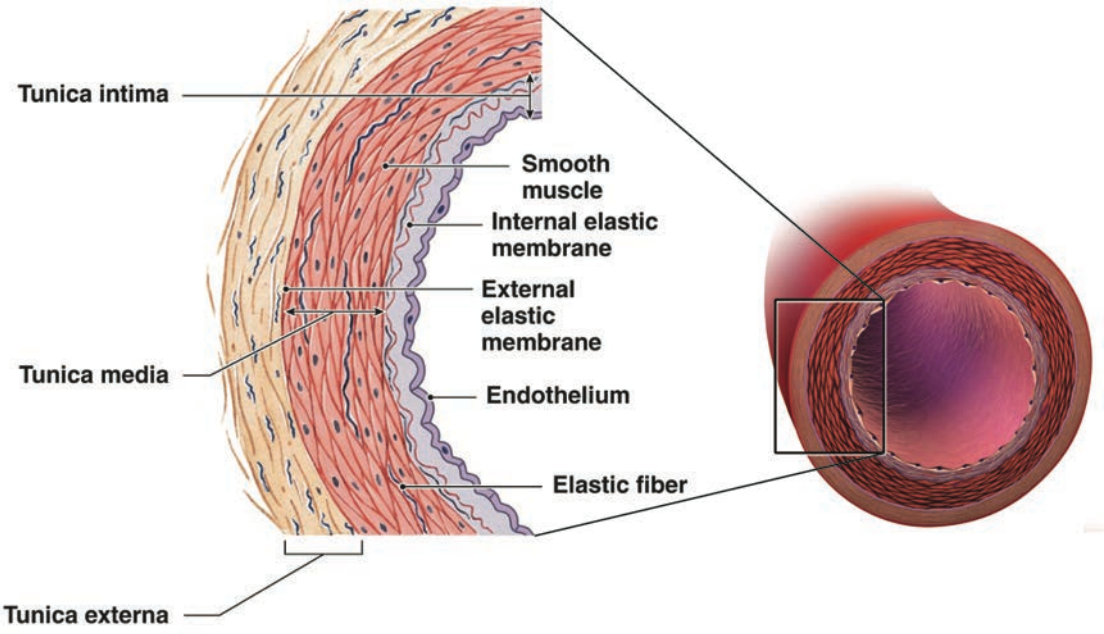

Figure 2. Structure of the vascular wall. *Adapted from Pearson Education, 2011 and 'Medical Gallery of Blouse Medical 2014'.*[10]

Function of the endothelium

The endothelium is a cell monolayer, which physically separates the blood from the rest of the vessel. As opposed to other tissues, it has the unique feature to maintain blood in a liquid state. The endothelium regulates constriction and dilatation of the vessel, proliferation, and migration of SMCs, and platelet adhesion and aggregation. It controls thrombogenesis and fibrinolysis via endogenous autacoids, nitric oxide, and lipid mediators, such as prostacyclin.[11] Additionally, it acts as a semipermeable barrier that controls the exchange of ions and macromolecules between blood and surrounding tissues via tight

junctions.[12] The endothelium also triggers the recruitment and extravasation of leukocytes, for example after tissue damage, via expression of cytokines and cell adhesion molecules.[13,14]

Endothelial Dysfunction

Endothelial dysfunction is an early hallmark of atherogenesis and can predict outcome in CVD.[15-17] However, it is now known that endothelial dysfunction can also be a result of thrombosis and it is still under debate, whether it is a true risk factor or rather a surrogate end point.[18,19] An important measure of endothelial function is its ability to trigger dilatation of vessels upon stimulation with acetylcholine (ACh).[20] The normal reaction of the endothelium in response to ACh is to release nitric oxide (NO), which causes relaxation of the SMCs of the tunica media.[21] In a dysfunctional endothelium, the ability to relax the vessel is impaired. This observation was first made in hypertensive rats and hypercholesterolemic rabbits and very soon thereafter in human atherosclerotic coronary arteries.[22-24]

While the endothelium can be stressed by many different factors, such as hypertension, atherosclerosis, hypercholesterolemia, diabetes, and obesity, all these stimuli may trigger endothelial dysfunction by an increase in reactive oxygen species (ROS).[25,26] ROS can be free radicals that possess unpaired electrons, like superoxide (O_2^-) or NO, or they can be compounds with oxidising effects, such as hydrogen peroxide (H_2O_2).[27] ROS are constantly produced in all cell types, mainly by mitochondrial proteins, xanthine oxidase, NADH/NADPH oxidase, and nitric oxide synthase (NOS).[27,28] In physiological conditions, ROS play important roles in various cell signalling processes and redox control.[26] The presence of O_2^- is regulated via a group of antioxidant enzymes called the superoxide dismutases, which catalyse the conversion of O_2^- into oxygen and H_2O_2. H_2O_2 is subsequently converted to water by catalase or glutathione peroxidase.[29,30] However, when the generation of ROS in the endothelium is increased by external stressors or can not be detoxified, these highly reactive compounds readily inflict oxidative damage on DNA, RNA, proteins, and lipids.[31]
In the endothelium, vasodilatation can be impaired by O_2^-, which decreases the bioavailability of NO by reducing it to peroxynitrite ($ONOO^-$), and inhibits guanylyl cyclase, a direct target of NO.[32] $ONOO^-$ furthermore also inhibits guanylyl cyclase, inactivates endothelial NOS (eNOS), increases levels of superoxide by inhibiting SOD, and promotes endothelial dysfunction by inhibiting prostacyclin synthase.[32] Of note, ROS also play a role in atherosclerosis

and thrombosis, for example via oxidising low-density lipoprotein (LDL) to oxidised LDL (oxLDL), as will be discussed in the following sections. In endothelial cells, the presence of oxLDL impairs NO production.[33]

4.3 Atherosclerosis

Atherosclerosis is a progressive disease that is characterised by a chronic vessel inflammation as well as accumulation of lipids and fibrous elements in large arteries.[34]

Initiation of atherosclerosis

The initiation of atherosclerosis is mediated by the endothelium, which is activated by risk factors as described above. Endothelial dysfunction results in leaky tight junctions that permit circulating apolipoprotein B-containing lipoproteins, especially LDL, to enter the subendothelial space in the vessel wall and promote intimal thickening.[35,36] Furthermore, LDL can be oxidised by ROS to form oxLDL. oxLDL induces endothelial expression of pro-atherothrombotic genes such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), Cyclooxygenase 2 (COX-2), and tissue factor (TF) and activates SMCs and macrophages.[37] While the endothelium would resist firm adhesion with leukocytes in healthy conditions, in a dysfunctional endothelium, ROS, inflammatory signals, and oxLDL trigger an increase in cellular adhesion molecules, which recruit leukocytes, especially monocytes and T lymphocytes (T-cells), to the endothelium.[38] Although VCAM-1 is most likely the predominant adhesion molecule (***Figure 3A***), triggering this effect due to its selectivity for monocytes and T-cells and its increased presence in endothelial cells at sites of early atheroma, also ICAM-1, P-selectin, and E-selectin play important roles.[39,40]

Leukocyte infiltration

Once leukocytes are bound to the endothelium, they enter the intima by diapedesis.[40] Initially, chemoattraction of leukocytes to the intima may be caused by oxLDL, which triggers apoptosis of SMCs via formation of ROS, which in turn signals phagocytic cells to clear the cell debris.[41,42] This depends mostly on monocyte chemoattractant protein-1 (MCP-1) and CXC chemokines, such as IP-10, Mig, and I-TAC, that are expressed by atheroma-associated cells, including the endothelium, SMCs and macrophages (***Figure 3***).[43-45]

Monocytes play a predominant role in the atherosclerotic lesion. Once inside the thickened intima, they develop characteristics of macrophages and start

endocytosis of not only dead cells, but also LDL and oxLDL. The uptake of lipoproteins is mediated by an increase in scavenger receptors, which mainly results from macrophage colony-stimulating factor (M-CSF) overexpression that increases cytokine and growth factor production in macrophages.[34,46] The accumulation of lipid droplets in their cytoplasm eventually transforms macrophages into foam cells.[47] These cells produce ROS and pro-inflammatory cytokines that amplify the inflammatory response in the plaque, matrix metalloproteinases (MMPs) that are able to destabilise the plaque by degrading extracellular matrix, and TF that triggers thrombotic complications upon plaque rupture (***Figure 3A***).[48-51]

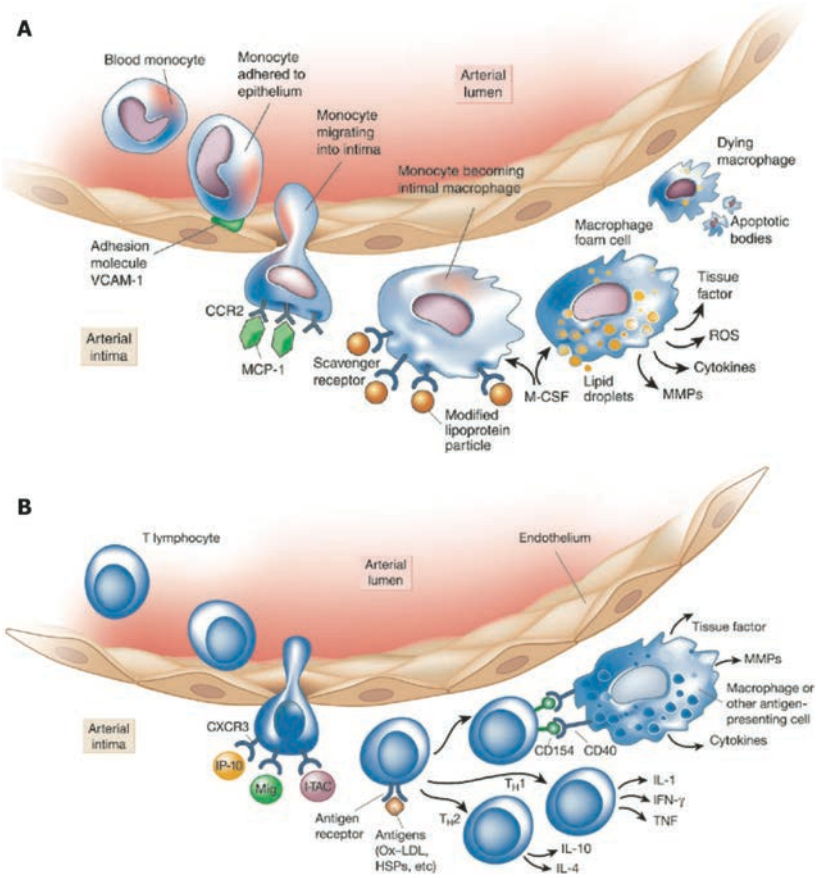

Figure 3. Leukocyte infiltration of the vascular intima and its effects in atherosclerosis. Adhesion molecules such as vascular cellular adhesion molecule 1 (VCAM-1) expressed by the activated endothelium facilitate leukocyte adhesion. **A**: Monocytes migrate into the intima following a gradient of monocyte chemoattractant protein 1 (MCP-1) that interacts with their C-C chemokine receptor type 2 (CCR2). Then, monocytes turn into macrophages that express scavenger receptors to ingest modified lipoprotein particles, such as oxLDL. Mediated by macrophage colony-stimulating factor (M-CSF) macrophages accumulate lipid droplets which give them characteristics of a foam cell, that is releasing ROS, expressing TF, matrix metalloproteinases (MMPs) and cytokines, and eventually undergoes apoptosis.
B: T lymphocytes are migrating into the intima following interactions of their C-X-C motif chemokine receptor 3 (CXCR3) with interferon-gamma (IFN-γ) inducible protein 10 (IP-10), monokine induced by gamma interferon (Mig), and interferon-inducible T-cell alpha chemoattractant (I-TAC). Antigens, such as oxLDL, cause the T-cells to activate macrophages and to differentiate into T helper cells (Th1 or Th2), that produce interleukins 1 (IL-1), 4 and 10, IFN-γ and tumour necrosis factor (TNF). *Adapted from Libby, Nature, 2002*.[34]

oxLDL not only affects macrophages but also stimulates plaque resident T-cells along with other antigens. These T-cells can then activate macrophages directly via CD40-CD154 interaction, or differentiate into T helper (Th) 1 or 2 cells, which further amplify the inflammation in the lesion by releasing cytokines (***Figure 3B***).[52-54]

Plaque progression and rupture

Stimulated by lipids and lipoproteins, macrophages and SMCs accumulate in the subendothelial layer of the arterial wall. Subsequently, they may undergo apoptosis or necrosis and form the necrotic core, which further enhances inflammation and recruitment of additional leukocytes.[55-58] These processes establish a vicious cycle that promotes progression and growth of an atherosclerotic lesion (***Figure 4***).

In an advanced atherosclerotic lesion, SMCs migrate to the intima where they form a collagen-rich fibrous cap that separates the atherosclerotic core from the vessel lumen and stabilises the plaque. This cap can rupture mediated by SMC-apoptosis and destabilising factors, such as MMPs, which subsequently exposes highly thrombogenic core material to the blood, causing thrombosis (***Figure 4***).[59,60]

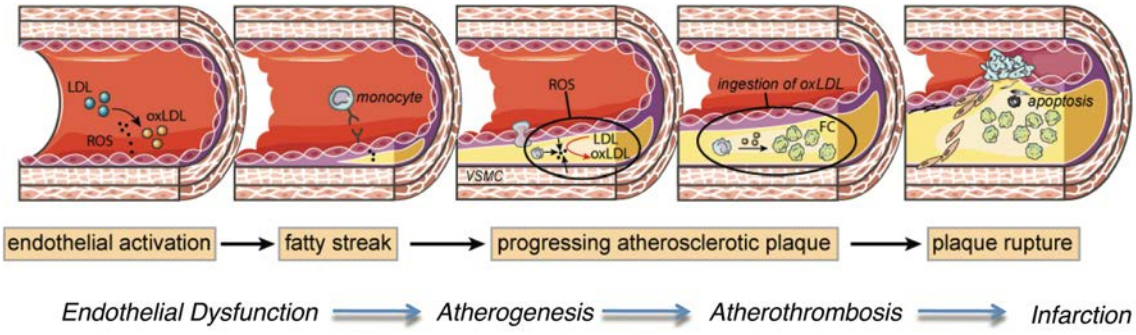

Figure 4. Development of endothelial dysfunction and progression to atherothrombosis. Excess reactive oxygen species (ROS) oxidise low-density lipoprotein (LDL) to oxLDL which transmigrates in the tunica intima, leading to endothelial activation. Circulating monocytes adhere to the activated endothelium, transmigrate into the subendothelial space and differentiate into macrophages. ROS, generated from endothelial cells, vascular smooth muscle cells (VSMCs) and macrophages further promote the oxidization of LDL particles, which are taken up by macrophages that in turn differentiate into foam cells (FC). Accumulating foam cells form fatty streaks. VSMCs migrate into the arterial intima forming a fibrous cap, which covers the lipid-rich core of the progressing atheroma. Apoptosis of plaque-resident cells contributes to fibrous cap thinning and eventual plaque rupture. *Figure created by Dr. Stephan Winnik, University Hospital Zurich*.

4.4 Arterial thrombosis

Blood coagulation is a central mechanism in wound repair that is indispensable to seal a damaged vessel, prevent blood loss, and initiate the healing process.[61] However, in occurrence of atherosclerosis, coagulation can be triggered pathologically by rupture of an atherosclerotic plaque or plaque erosion, which is characterised by an absence of the endothelium.[62,63] In these cases, coagulation may lead to thrombosis of an artery. Arterial thrombosis is the occlusion of an artery by a blood clot and hence an obstruction of oxygen and nutrient flow to the adjacent tissues. It is the major complication in CVD and may lead to stroke or myocardial infarction.[64]

Tissue factor and the coagulation cascade

The coagulation cascade consists of a number of inactive soluble factors in the blood that subsequently activate each other to generate clot stabilising fibrin molecules and thrombin, which initiates platelet aggregation. A central activator of the coagulation cascade is tissue factor (TF). TF is a protein of 47 kDa that is constitutively expressed in cells of the adventitia and SMCs of the media, while its expression is latent but can be induced in leukocytes and the endothelium.[65-67] Originally, the endothelium was thought to be a natural barrier separating TF in the inner vascular wall from the other coagulation factors in the blood stream, but it is now known that TF also exists in the blood in form of latent TF microparticles and as an alternatively spliced soluble form.[68-71]

Once active TF gets in contact with blood it binds to coagulation factor VII (FVII), which is subsequently activated to FVIIa. The interaction of TF and FVII initiates the extrinsic coagulation cascade (***Figure 5***). The TF:FVIIa complex activates FX and FIX. FIXa initiates the intrinsic coagulation cascade, which further amplifies this process by forming a complex with FVIIIa that also activates FX. FXa binds to FVa and the FXa:FVa complex cleaves prothrombin to thrombin, which plays a central role in the coagulation cascade.[72] Thrombin activates platelets by cleaving protease-activated receptors, cleaves fibrinogen to soluble fibrin monomers, and activates FXIII, which links these monomers to a clot-stabilising fibrin polymer (***Figure 5***).[73,74] Furthermore, thrombin enhances the intrinsic pathway by activating FXI, which in turn activates FIX.[75,76] More recently, coagulation factor FXII, that had been thought to have no function in coagulation *in vivo*, has been identified as an important initiator of the intrinsic pathway, as it is able to activate FXI.[77]

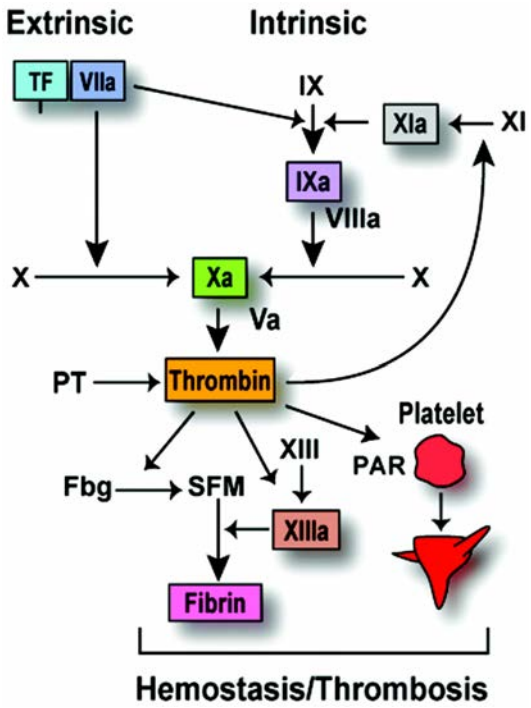

Figure 5. The coagulation cascade. Formation of the TF:FVIIa complex initiates clotting by activating FX and FIX. Alternatively, FXI can activate FIX. The prothrombinase complex (FVa:FXa) activates prothrombin (PT). Thrombin activates various proteases and cofactors. Thrombin cleaves fibrinogen (Fbg) to soluble monomers (SFM), which are cross-linked by FXIIIa, and activates protease-activated receptors (PARs) on platelets, which leads to the formation of a clot. *From Mackman et al., ATVB, 2007.*[72]

To keep homeostasis in physiological conditions, the coagulation cascade is regulated by a number of inhibitors. Among the most important regulators are TF pathway inhibitor (TFPI) which inhibits the TF:FVIIa complex and FXa; Protein C which is inhibiting FVa and FVIIIa; and antithrombin which is inhibiting thrombin, FXa, FIXa and FVIIa.[78-80]

Platelets

Platelets are fragments of megakaryocytes without a nucleus that are shed in the process of megakaryocyte maturation either in bone marrow or the lung.[81] The life cycle of a platelet is limited to 5-7 days during which it decreases in size. After this time, or after activation and incorporation into a blood clot, platelets are cleared by neutrophils and macrophages and disposed of via the spleen.[82] Platelets possess a characteristic receptor-rich cell membrane and granules that contain adhesion molecules and platelet agonists.

In healthy conditions, the endothelium and platelets do not favour adhesion. Both cell types are negatively charged and the endothelium produces prostacyclin and NO, which raise cyclic adenosine monophosphate (cAMP) and

cyclic guanosine monophosphate (cGMP) levels in platelets.[83,84] cAMP and cGMP stimulate protein kinases A and C to phosphorylate platelet agonist receptors, which keeps platelets inactivated.

However, the subendothelial matrix contains many proteins, which are able to activate platelets and facilitate binding. Upon vascular injury, such as the rupture of an atherosclerotic plaque, the subendothelial matrix is exposed to platelets in the blood. Platelet binding to the vascular wall is then facilitated most prominently by glycoprotein and integrin receptor binding to collagen and von Willebrand factor (vWF).[82] Firm adhesion, which leads to a flattened shape of the platelets, is additionally catalysed by atherosclerosis associated endothelial dysfunction, which limits the availability of the physiological platelet inhibitors prostacyclins and NO.[85] Secondary to firm adhesion of platelets is their activation by agonists, such as collagen, ADP, Thromboxane A_2, and the most potent platelet activator thrombin (***Figure 6***).[86]

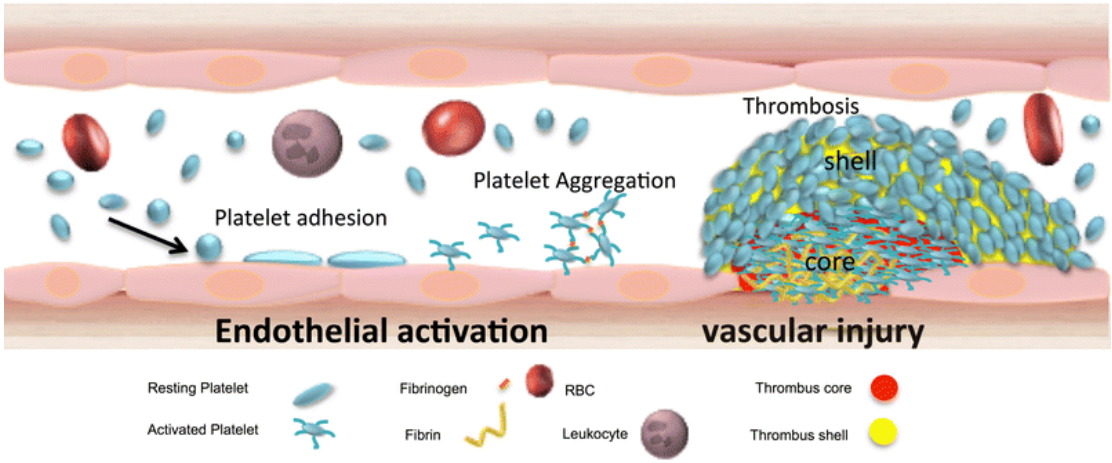

Figure 6. Role of platelets in haemostasis and thrombosis. Upon endothelial activation or damage, platelets are able to bind to the endothelium or the subendothelial matrix. This increasingly takes place upon vascular injury, where matrix-derived proteins, such as collagen, are exposed to the blood. Once a platelet gets activated, its shape shifts and it recruits more platelets that begin to aggregate and form a thrombus. RBC: Red blood cell. *Adapted from Holinstat et al., Cancer Metastasis Rev, 2017*.[82]

Activated platelets undergo key structural changes induced by an agonist-mediated increase in calcium levels, and increase their surface area approximately by 4-fold. Additionally, activated platelets externalise all contents of their granules, which are laden with platelet adhesion molecules, such as vWF, p-selectin, and fibrinogen, as well as platelet agonists, mostly ADP. These proteins recruit more platelets and cause them to aggregate. The platelets are further linked to each other and stabilised by fibrinogen and fibrin molecules from the coagulation cascade.[87] Finally, activated platelets also provide an effective catalytic surface for the activation of the coagulation cascade.[88]

4.5 Neutrophils in Atherothrombosis

This chapter is based on:
Gaul DS, Stein S, Matter CM.
Neutrophils in cardiovascular disease.
Eur Heart J 2017; **38**(22): 1702-4

As leukocytes are involved in inflammatory responses of the body, it is no surprise that they are also involved in chronic inflammatory diseases, like atherosclerosis. In the past, many atherothrombosis studies focused especially on the role of monocyte/macrophages and T-cells. However, more recently, neutrophils have gained a lot of interest and emerged as intriguing new players in atherothrombosis.[89]

Normal neutrophil function

Neutrophils are polymorphonuclear leukocytes that form the initial defence against pathogens and protect the host by mediating inflammatory and innate immune responses.[90] Neutrophils have developed distinct mechanisms to be able to defend their host: phagocytosis, apoptosis, externalisation of anti-pathogenic granule content, release of ROS, and formation of neutrophil extracellular traps (NETs).[91] These powerful immune responses can be triggered by pathogens, such as bacterial lipopolysaccharide (LPS), cytokines, and other inflammatory cells or stimuli. However, they can be detrimental in a disease context.

Neutrophils in atherosclerosis

Neutrophils are recruited to an atherosclerotic lesion by macrophage-derived chemokines and transmigrate into the lesion via oxLDL-dependent upregulation of ICAM-1 and increased contractility of endothelial cells.[92-94]

Once inside a lesion, the neutrophils start to react to the ongoing inflammation with the intention to resolve it, but eventually they worsen the outcome. Degranulated proteins recruit more monocytes by facilitating adhesion to the endothelium, promote plaque instability by breaking down collagen, and catalyse lipoprotein oxidation.[95-99] The same effects are caused by release of ROS.[100] Finally, neutrophils in the atheroma readily undergo apoptosis and thus release signals that yet again recruit monocyte/macrophages into the plaque (***Figure 7***).[101,102]

Neutrophils in thrombosis and ischaemia-reperfusion injury

In endothelial damage-mediated thrombosis, neutrophils have been shown to initiate thrombus formation by being the first cell-type physically present at the site of damage and providing TF to trigger coagulation.[67] The neutrophil-derived proteinases cathepsin G and elastase furthermore degrade TFPI, the main inhibitor of the extrinsic coagulation pathway.[103] Finally, ROS released by neutrophils at the site of thrombus formation can activate platelets (***Figure 7***).[104]

Neutrophils do not only aggravate thrombotic processes, they also play an important role in the processes following the dissolution of a thrombotic occlusion. If a coronary artery is occluded, a quick reperfusion is essential to save the myocardial tissue from ischaemia.[105] After reperfusion, neutrophils are recruited to the infarcted tissue by dying cells and damaged extracellular matrix, where they clear dead cells and recruit monocytes that degrade the damaged matrix.[106] While this is initially beneficial, secondary effects by activated neutrophils, such as degranulation and ROS release, damage intact cells and extracellular matrix, which may increase infarct size. Indeed, reperfusion with neutrophil-deprived blood reduced injury in dogs and reperfusion injury is associated with recurrent atherosclerosis in mice and patients.[107-109]

Neutrophil extracellular traps and cardiovascular disease

NETs have only recently gotten into focus of research, but already now it is clear that NET formation occurs in a remarkable number of diseases where neutrophils are involved, indicating that its importance may have been overlooked in the past.[89]

NETs consist of externalised neutrophil DNA and granular proteins, which enable them to kill pathogens.[110] They can be triggered by pathogen-derived endotoxins, like LPS, or by P-selectin expressed in activated platelets and endothelial cells.[111,112]

There are currently two models of NETs discussed: NETs containing nuclear DNA that are released during a programmed cell death, and NETs containing mitochondrial DNA that can be released by viable neutrophils.[113,114] While both mechanisms of NET formation may exist, both hypotheses agree that the formation of NETs depends on the generation of ROS.[115,116]

NETs have been identified in human atherosclerotic lesions, where they promote atherogenesis and are associated with a severe atherothrombotic state, likely

due to release of granular proteins, ROS and pro-thrombotic factors (***Figure 7***).[117,118] Furthermore, NETs can be induced by the activated endothelium and are susceptible to NET-mediated cell death.[112]

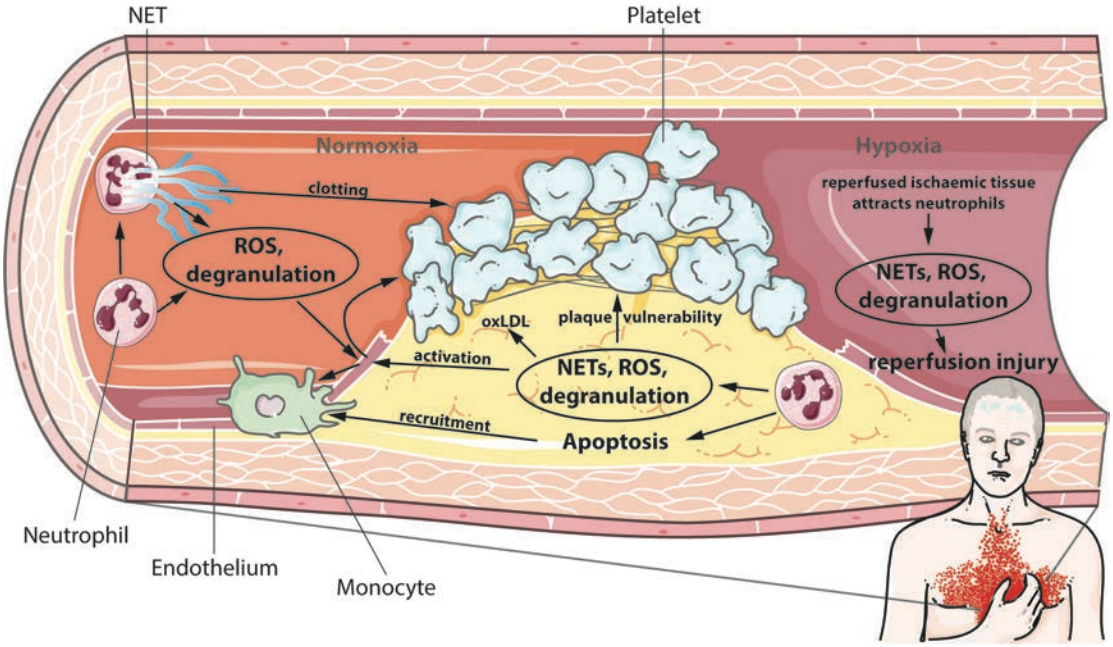

Figure 7. Effects of neutrophils in atherosclerosis, thrombosis, and ischaemia-reperfusion injury. NETs: neutrophil extracellular traps; ROS: reactive oxygen species; oxLDL: oxidised low-density lipoprotein. *From Gaul et al., Eur Heart J, 2017.*[89]

NETs demonstrate strong pro-thrombotic properties. Indeed, the use of DNAse I to degrade NETs in animals reduced thrombosis, myocardial infarction, and ischemic stroke.[119-122] Importantly, NETs also occur in human coronary thrombi, where they are associated with coronary infarct size.[123-125] When neutrophils release DNA, this huge molecule acts as a surface that binds TF, FXII, and granular proteins, like TFPI inhibitors cathepsin G or elastase, and thus activates both the extrinsic as well as the intrinsic coagulation pathway.[103,126,127] Furthermore, NETs bind vWF, fibronectin, and fibrinogen, all of which are triggering platelet binding and aggregation.[112,128]

Finally, NETs are also implicated in ischaemia-reperfusion injury in rats and degradation of NETs is discussed to be therapeutically used to decrease the severity of this injury (***Figure 7***).[129]

4.6 Sirtuins - mediators of caloric restriction

Silent information regulator 2 proteins (sirtuins) are a protein family consisting of seven members (Sirt1-7) that are highly conserved between species and occur in different cellular compartments (***Figure 8A***).[130] All sirtuins exhibit protein

deacetylase activity, but some of the members also act as ADP-ribosylases and/or deacylases of succinyl, malonyl, glutaryl or long-chain fatty acyl groups.[131] Regardless of their functions, all sirtuins are dependent on the cofactor NAD^+ and, consequently, sirtuin activity is increased in times of low nutrient availability (***Figure 8B***).[132] Interestingly, caloric restriction has been shown to prolong life span and delay onset of age-related diseases in many species, including mammals.[133-135] When first studies in yeast showed that many of these effects may be mediated by the sirtuins in 1999, many researchers took an interest in deciphering the underlying molecular mechanisms.[136] It is now known that sirtuins possess many beneficial roles in survival and aging (***Figure 8B***), and some of them have been associated with longevity in mice and humans.[137,138] To date, the only efficient way to activate sirtuins is by caloric restriction.[139]

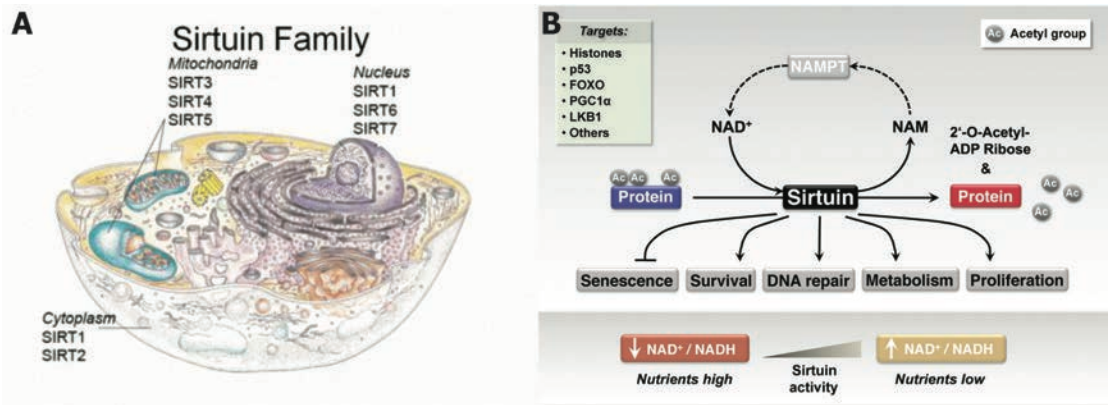

Figure 8. The sirtuin family of protein deacetylases. **A**: The sirtuin family consists of seven members that are distributed in different cell compartments. While Sirt2 is found in the cytoplasm, Sirt1 can be both located in cytoplasm and the nucleus. Sirt6 and Sirt7 are nuclear proteins and Sirt3-5 are located in the mitochondria. **B:** Sirtuins are NAD^+-dependent deacetylases that target histone and nonhistone proteins (left upper box) to regulate a wide range of cellular functions such as cellular senescence, survival, DNA repair, metabolism, and cell cycle progression. Because sirtuins require NAD^+ for their catalytic activity, their enzymatic activity is higher in situations of energy distress. *Figure 8A was retrieved from the Denu Lab at the University of Wisconsin-Madison (http://devriesgen677s09.weebly.com/sirtuin-family.html) and Figure 8B was adapted from Oellerich et al., Circ Res, 2012*.[140]

Sirtuins in cardiovascular disease

Sirtuins take over particularly interesting roles in CVD, as they are positively influencing a number of cardiovascular risk factors, such as the metabolic syndrome.[141,142] Metabolic syndrome is triggered by high-caloric diets and physical inactivity and is characterised by the combination of high blood pressure, obesity, inflammation, glucose intolerance, and dyslipidaemia, all of which are associated with development of CVD and diabetes.[143] Sirt1, the best-

characterised member of the sirtuins, for instance, is able to improve glucose tolerance and lipid homeostasis and thus confers atheroprotection.[144-146] However, sirtuins are also directly involved in endothelial function, atherosclerosis, and thrombosis.[141] This dissertation is focusing on the role of Sirtuin 3 and Sirtuin 6 in CVD.

Sirtuin 3 in cardiovascular disease

Sirtuin 3 (Sirt3) is a mitochondrial deacetylase, which affects acetylation of hundreds of mitochondrial proteins and thereby maintains mitochondrial function and homeostasis.[131,147-149]

In times of low energy, NAD^+ levels rise and activate Sirt3, which in turn deacetylates and thus activates long-chain acyl CoA dehydrogenase (LCAD), acetyl coenzyme A-synthetase 2 (AceCS2), glutamate dehydrogenase (GDH), and isocitrate dehydrogenase 2 (IDH2) (***Figure 9***).[150-152] LCAD and AceCS2 generate acetyl coenzyme A (Acetyl-CoA) via oxidation of fatty acids and conversion of acetate, respectively.[150,151] GDH and IDH2 are involved in the generation of α-ketoglutarate.[152-154] Both acetyl-CoA and α-ketoglutarate are important substrates in the tricarboxylic acid (TCA) cycle and hence activate it, which leads to improved regeneration of NADH from NAD^+. NADH is needed to fuel the oxidative phosphorylation cascade to generate energy in form of adenosine triphosphate (ATP).

Sirt3 is not only indirectly providing NADH for the oxidative phosphorylation, it is also able to activate complex I, III and V of this cascade directly, and complex II via deacetylation of succinate dehydrogenase (SDH) (***Figure 9***).[155-158] Again this leads to an increased generation of ATP and the cell can keep up energy production in absence of nutrients with these mechanisms.

During the synthesis of ATP in the oxidative phosphorylation cascade, ROS are generated as by-products.[159] In fact, mitochondria are a major source of cellular ROS, as approximately 1-2% of all oxygen that is consumed by oxidative phosphorylation is converted to superoxide (O_2^-) due to leakage.[160] Superoxide dismutase 2 (SOD2) is the main scavenger of O_2^- in mitochondria. It directly converts O_2^- to hydrogen peroxide (H_2O_2), which is furthermore converted to water by catalase (CAT) or glutathione peroxidase.[29,30] Sirt3 stimulates this process by activating transcription factor Foxo3a, which upregulates expression of SOD2 and CAT (***Figure 9***).[161] Moreover, Sirt3 deacetylates SOD2 directly at

lysine (K) 58, 89 and 122, which are adjacent to the active site of SOD2, and thus increases its enzymatic activity and capacity to scavenge O_2^{-}.[162,163]

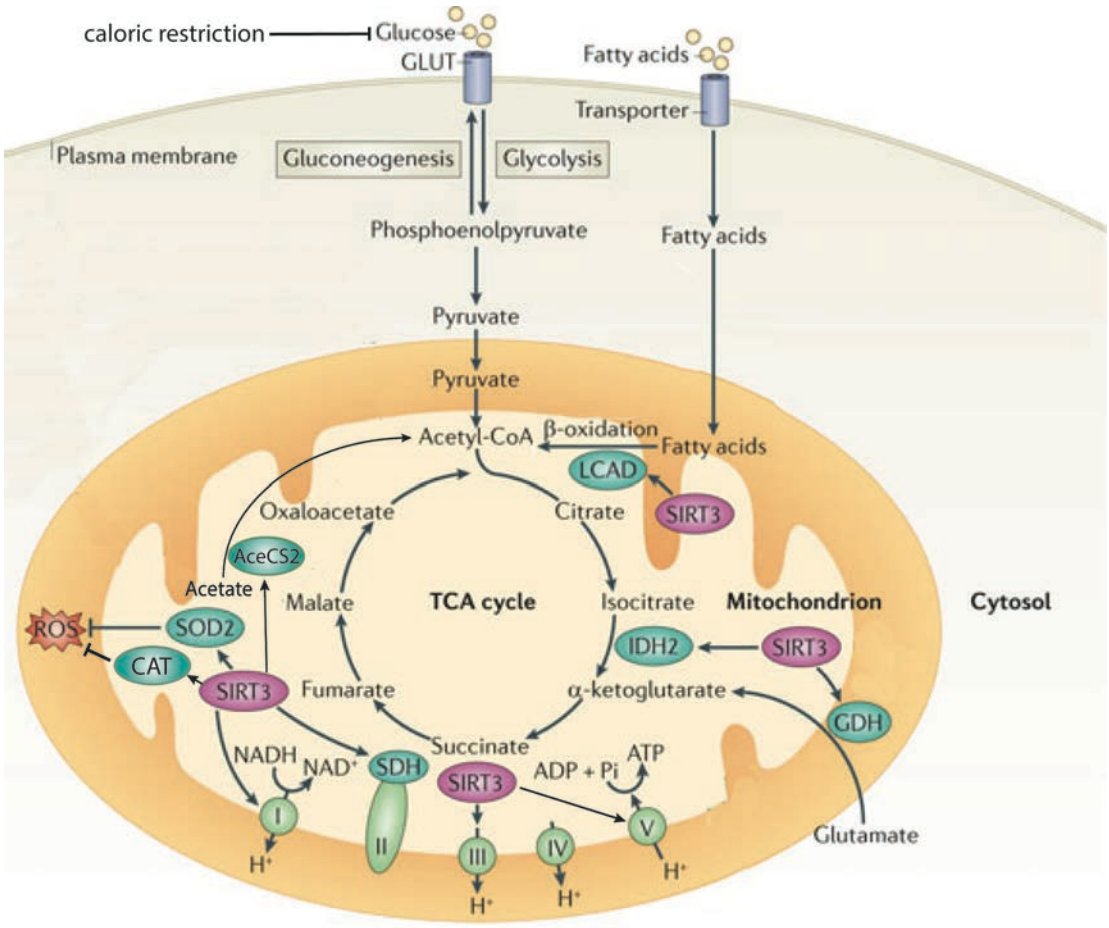

Figure 9. The role of Sirt3 in mitochondrial homeostasis. Sirt3 is maintaining mitochondrial homeostasis in low energy conditions on three levels: (1) By activating enzymes that generate substrates for the tricarboxylic acid (TCA) cycle, it is facilitating regeneration of reduced nicotinamide adenine dinucleotide (NADH); (2) By direct and indirect activation of complexes I, II, III, and V it is increasing oxidative phosphorylation and generation of adenosine triphosphate (ATP); (3) By activating superoxide dismutase 2 (SOD2) and transcriptionally upregulating SOD2 and Catalase (CAT), it is scavenging reactive oxygen species (ROS). GLUT: glucose transporter; LCAD: long-chain acyl CoA dehydrogenase; AceCS2: acetyl coenzyme A-synthetase 2; IDH2: isocitrate dehydrogenase 2; GDH: glutamate dehydrogenase; SDH: succinate dehydrogenase; ADP: adenosine diphosphate; P_i: inorganic phosphate. *Adapted from Houtkooper et al., Nat Rev Mol Cell Biol, 2012*.[145]

An excess of ROS in the mitochondria may induce mitochondrial dysfunction and apoptosis, leading to ageing and age-related diseases.[28] Consequently, deletion of Sirt3 in mice accelerates development of diabetes, metabolic syndrome, and age-related hearing loss, and induces pulmonary artery hypertension.[153,156,164,165] Interestingly, these effects in Sirt3-depleted mice are only obvious, if an additional stressor, such as a chronic high fat diet is used to challenge the system.[148,164] Along these lines, Sirt3 is able to prevent cardiac hypertrophy by decreasing ROS levels via SOD2 and CAT, and by regulating the mitochondrial

permeability transition pore to prevent mitochondrial dysfunction.[161,166] Sirt3 also protects cultured endothelial cells from mitochondrial ROS and cardiomyocytes from stress-induced apoptosis.[167,168] Finally, Sirt3 has been associated with longevity in humans.[137,169]

Prior to the work presented in this dissertation, the role of Sirt3 in atherosclerosis, endothelial function, and arterial thrombosis had not been investigated.

Sirtuin 6 in cardiovascular disease

Sirtuin 6 (Sirt6) is a nuclear-located sirtuin that acts as deacetylase, deacylase of long-chain fatty acyl groups, and as ADP-ribosyltransferase.[170] Sirt6 exerts a wide range of effects on metabolism, inflammation, and ageing. Indeed, a constitutive global deletion of Sirt6 in mice leads to an aging-like phenotype and severe hypoglycaemia, which causes 60% of the animals to die at an age of approximately 4 weeks, and the rest within 1 year.[171,172] Overexpression of Sirt6, on the other hand, is able to increase lifespan in male mice and protects from consequences of diet-induced obesity.[173] Of Sirt6's molecular functions, the deacetylation of lysines of histone 3 (H3) are especially well characterised and to date, the lysines (K) 9, 18 and 56 of H3 have been identified as deacetylation targets.[174-176]

Sirt6 mediates glucose homeostasis via H3 deacetylation-dependent inhibition of expression of HIF1α-dependent glycolytic genes, and via direct acetylation of general control non-repressed protein 5 (GCN5), which controls hepatic gluconeogenesis.[177,178] In the same fashion, Sirt6 acts as a tumour suppressor by inhibiting aerobic glycolysis in tumour cells.[179] Sirt6 is furthermore implicated in tumour suppression, as it represses NF-κB-mediated gene-transcription of survivin- and c-MYC-mediated ribosomal biogenesis in cancer cells (***Figure 10***).[179,180] Interestingly in some studies, Sirt6 has been shown to play an oncogenic role.[181,182] This is likely due to the function of Sirt6 as a keeper of genomic stability.[170]

Sirt6 plays an important role in the maintenance of genomic stability by affecting telomere structure and function and by mediating DNA repair. Via deacetylation of H3K9 and H3K56, Sirt6 facilitates the proper association of Werner syndrome protein (WRN) with telomeric chromatin and hence regulates adequate capping of telomeres.[174,183,184] Additionally, Sirt6 is involved in DNA repair, especially double-strand break (DSB) repair. It ADP-ribosylates poly [ADP-ribose]

polymerase 1 (PARP1), a protein mediating base excision and DSB repair.[171,185] Furthermore, it protects from DSB and improves homologous recombination, by deacetylating C-terminal binding protein interacting protein (CtIP), stabilises DNA-dependent protein kinase at chromatin for DSB repair, and recruits sucrose nonfermenting 2 homologue (SNF2H) to DNA-break sites to mediate repair.[186-188] These observations highlight a beneficial role of Sirt6 in ageing, which is furthermore supported by the notion that Sirt6 can translocate into the cytoplasm in stress conditions where it promotes dephosphorylation of Ras-GAP SH3 domain binding protein (G3BP), which regulates structure and dynamics of stress granules (***Figure 10***).[189]

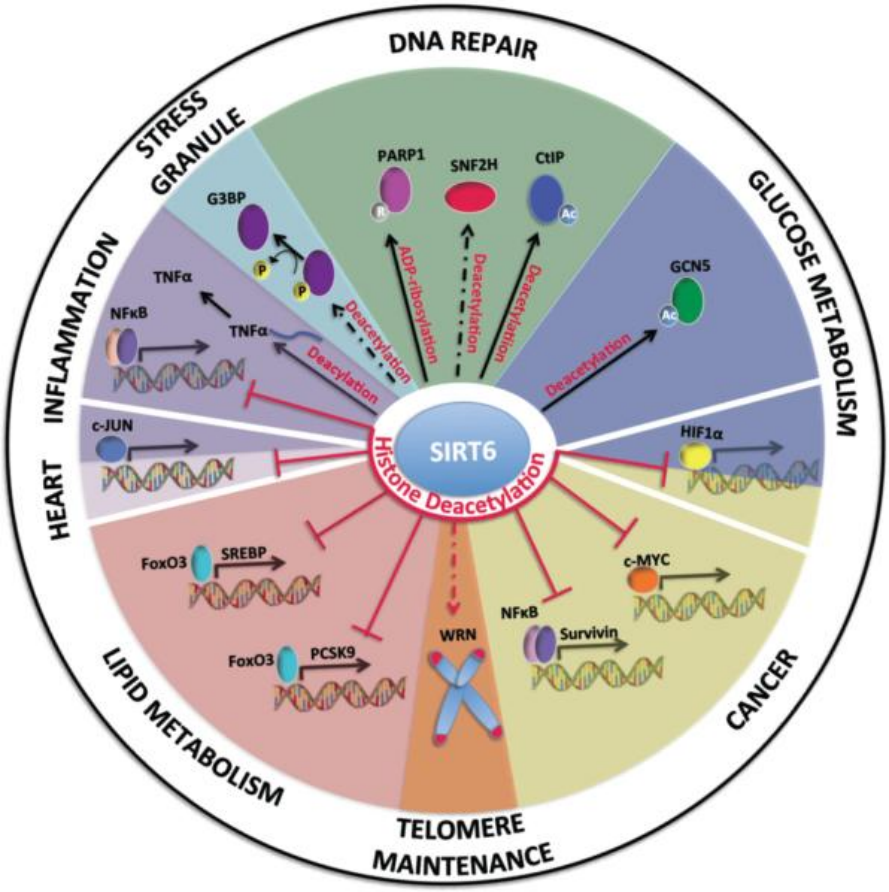

Figure 10. Sirt6 cellular functions and their impact on organismal biology and disease. Sirt6 primarily functions as an H3K9 and H3K56 histone deacetylase that decreases chromatin accessibility for transcription factors such as nuclear factor kappa (NF-κB) or c-JUN to their respective promoters and thus inhibits expression of their target genes. Sirt6 can also regulate protein activity through direct or indirect deacetylation, deacylation, and ADP-ribosylation. Via these mechanisms, Sirt6 is mediating stress response, DNA repair, glucose metabolism, cancer, telomere maintenance, lipid metabolism, and inflammation. Solid arrow: Sirt6 directly modifies the protein or directly affects histone deacetylation at the promoters of target genes. Dashed arrow: Sirt6 deacetylation activity is necessary, but is not direct. Red arrows: Histone deacetylation. P (phosphorylation), Ac (acetylation) and R (ADP-ribosylation). *Adapted from Kugel et al., Trends Biochem Sci, 2014.*[170]

Sirt6 also has beneficial functions in preventing the development of metabolic CVD risk factors. Not only may it be a therapeutic target to improve diabetes due to its involvement in glucose metabolism, it also positively affects blood lipid levels.[190-192] By deacetylation of H3K9 and H3K56, Sirt6 inhibits FoxO3-dependent expression of proprotein convertase subtilisin/kexin type 9 (PCSK9) gene, which is promoting degradation of LDL-receptor, and of the sterol-regulatory element binding protein (SREBP) gene, a key regulator of cholesterol biosynthesis (***Figure 10***).[191-193]

Sirt6 can also regulate inflammatory responses and, depending on the context, acts pro- or anti-inflammatory. Acylating a long-chain fatty acyl lysine, Sirt6 can catalyse the hydrolysis of lysine K19 and K20 of tumour necrosis factor-α (TNF-α), triggering TNF-α secretion from the cell.[194] TNF-α is an important pro-inflammatory mediator that triggers transcription of NF-κB- and AP-1-dependent pro-inflammatory genes. On the other hand, Sirt6 is able to inhibit exactly these two transcription factors. By association with NF-κB subunit RelA (p65) and AP-1 subunit c-JUN, Sirt6 deacetylates H3K9 and represses the activity of NF-κB- and AP-1 (***Figure 10***).[195,196]

The interaction of Sirt6 and c-JUN is also thought to protect from cardiac hypertrophy and heart failure.[197,198] In a mouse model of atherosclerosis, deletion of one Sirt6-allele caused endothelial dysfunction and increased atherosclerosis.[199] *In vitro* studies suggest, that these effects are triggered by increased expression of VCAM-1 and induction of pro-inflammatory cytokines via NF-κB in endothelial cells.[199,200]

However, the role of Sirt6 in arterial thrombosis remains elusive.

5 Hypotheses and research aims

Having in mind the roles of Sirt3 and Sirt6 in metabolic syndrome, ageing, and stress, we hypothesised that these two proteins are protective in the development and propagation of CVD. Therefore, we aimed to investigate the effect of loss of Sirt3 on atherosclerosis, endothelial dysfunction, and arterial thrombosis. Because the role of Sirt6 in endothelial function and atherosclerosis was already under investigation, we focused on determining the effect of endothelial specific loss of Sirt6 on arterial thrombosis.

5.1 The role of Sirt3 in atherothrombosis

Hypothesis

Loss of Sirt3 accelerates atherosclerosis, causes endothelial dysfunction, and increases arterial thrombosis by impairing Sirt3-mediated anti-oxidant defence mechanisms.

Specific aims

Sirt3 in atherosclerosis

a. Characterisation of the effect of Sirt3 deficiency on atherosclerosis in LDL-receptor knockout mice fed a high-cholesterol diet
b. Assessment of the effect of Sirt3 deficiency on systemic oxidative stress and metabolism in LDL-receptor knockout mice fed a high-cholesterol diet

Sirt3 in endothelial function

c. Evaluation of the effect of loss of Sirt3 on endothelial function in mice fed a high-cholesterol diet
d. Unravelling of the molecular effect of Sirt3 deficiency on cultured human aortic endothelial cells

Sirt3 in arterial thrombosis

e. Assessment of the effect of loss of Sirt3 on arterial thrombosis in mice stressed with bacterial lipopolysaccharide
f. Characterisation of the cell type and mechanism by which Sirt3 may influence arterial thrombosis

5.2 The role of Sirt6 in arterial thrombosis

Hypothesis

Endothelium-specific loss of Sirt6 promotes thrombosis by activating NF-κB and AP-1 mediated pro-inflammatory pathways in endothelial cells.

Specific aims

a. Generation and characterisation of endothelium-specific Sirt6 knockout mice
b. Evaluation of the effect of endothelial loss of Sirt6 on arterial thrombosis
c. Assessment of the molecular effects of Sirt6 deficiency on human aortic endothelial cells

6 Results

6.1 Deletion of Sirt3 does not affect atherosclerosis but accelerates weight gain and impairs rapid metabolic adaptation in LDL receptor knockout mice: implications for cardiovascular risk factor development

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Status of the manuscript:

Published in:	Basic Research in Cardiology 2014 Jan; 109(1): 399.
Published online:	27 December 2013
DOI:	10.1007/s00395-013-0399-0
PMID:	24370889
License:	Open access article under the terms of the Creative Commons Attribution License

Author contributions Daniel S. Gaul:

- Planning, data acquisition, analysis, statistical evaluation, and interpretation of the revision experiments for Basic Res Cardiol.
- Contributions to the individual figures:
    - Figure 4: contributed subfigures 4B, C, D, E, F, G and I
    - Supplemental Figure 1 (S1): contributed the whole figure
    - Supplemental Figure 2 (S2): contributed the whole figure
    - Supplemental Figure 3 (S3): contributed the whole figure
- Editing and proofreading of the manuscript

Basic Res Cardiol (2014) 109:399
DOI 10.1007/s00395-013-0399-0

ORIGINAL CONTRIBUTION

Deletion of Sirt3 does not affect atherosclerosis but accelerates weight gain and impairs rapid metabolic adaptation in LDL receptor knockout mice: implications for cardiovascular risk factor development

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Received: 31 May 2013 / Revised: 7 November 2013 / Accepted: 16 December 2013 / Published online: 27 December 2013

Abstract Sirt3 is a mitochondrial NAD^+-dependent deacetylase that governs mitochondrial metabolism and reactive oxygen species homeostasis. Sirt3 deficiency has been reported to accelerate the development of the metabolic syndrome. However, the role of Sirt3 in atherosclerosis remains enigmatic. We aimed to investigate whether Sirt3 deficiency affects atherosclerosis, plaque vulnerability, and metabolic homeostasis. Low-density lipoprotein receptor knockout ($LDLR^{-/-}$) and *LDLR/Sirt3* double-knockout ($Sirt3^{-/-}LDLR^{-/-}$) mice were fed a high-cholesterol diet (1.25 % w/w) for 12 weeks. Atherosclerosis was assessed en face in thoraco-abdominal aortae and in cross sections of aortic roots. Sirt3 deletion led to hepatic mitochondrial protein hyperacetylation. Unexpectedly, though plasma malondialdehyde levels were elevated in *Sirt3*-deficient mice, Sirt3 deletion affected neither plaque burden nor features of plaque vulnerability (i.e., fibrous cap thickness and necrotic core diameter). Likewise, plaque macrophage and T cell infiltration as well as endothelial activation remained unaltered. Electron microscopy of aortic walls revealed no difference in mitochondrial microarchitecture between both groups. Interestingly, loss of Sirt3 was associated with accelerated weight gain and an impaired capacity to cope with rapid changes in nutrient supply as assessed by indirect calorimetry. Serum lipid levels and glucose tolerance were unaffected by Sirt3 deletion in $LDLR^{-/-}$ mice. Sirt3 deficiency does not affect atherosclerosis in $LDLR^{-/-}$ mice. However, Sirt3 controls systemic levels of oxidative stress, limits expedited weight gain, and allows rapid metabolic adaptation. Thus, Sirt3 may contribute to postponing cardiovascular risk factor development.

Electronic supplementary material The online version of this article (doi:10.1007/s00395-013-0399-0) contains supplementary material, which is available to authorized users.

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Keywords SIRTUIN 3 · Atherosclerosis · Metabolism · Oxidative stress

Abbreviations

HDAC	Histone deacetylase
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
ROS	Reactive oxygen species
TCA cycle	Tricarboxylic acid cycle
MnSOD	Manganese superoxide dismutase
Cat	Catalase
IDH2	Isocitrate dehydrogenase 2
ORO	Oil red O
VCAM-1	Vascular adhesion molecule-1
LDLR	Low-density lipoprotein receptor
MCP-1	Monocyte chemoattractant protein-1
IL-1b	Interleukin 1 beta
TNFα	Tumor necrosis factor alpha
MDA	Malondialdehyde
Ad lib	Ad libitum
WAT	White adipose tissue
RER	Respiratory exchange ratio

Introduction

Sirtuins are a family of class III histone deacetylases (HDACs) that distinguish themselves from other HDAC classes by their dependency on nicotinamide adenine dinucleotide (NAD^+) as a cofactor [16]. Sirtuin activity is thus tightly linked to cellular energy levels, confining their activity to times of caloric restriction, the only regimen known to extend life span [12], and delay the onset of age-related diseases, including atherosclerosis and myocardial infarction [6, 21].

Among seven mammalian homologues Sirt3 is the only sirtuin that has been associated with *human* longevity and aging in health [3, 11, 30]. A distinct variability in the evolutionary conserved domain of the Sirt3 gene increased the genotype-specific survival function in a homogeneous population in southern Italy [30]. Accordingly, the presence of an intronic enhancer of the Sirt3 gene was observed to be associated with survival at old ages [3]. Finally, Sirt3 showed the highest degree of genetic variation among 24 candidate genes of aging in a population of healthy seniors aged 85 years and above, who had never been diagnosed with cancer, diabetes, cardiovascular, pulmonary or Alzheimer disease [11].

Together with Sirt4 and Sirt5, Sirt3 is targeted to the mitochondrial matrix, where—through deacetylation of a variety of substrates—it orchestrates mitochondrial oxidative metabolism and controls reactive oxygen species (ROS) homeostasis [1, 14, 23, 40]. In diverse contexts Sirt3 has been reported to regulate the tricarboxylic acid cycle and oxidative phosphorylation [1, 5, 8, 31, 33, 43]. An inevitable byproduct of these catabolic reactions is the generation of ROS. Intriguingly, Sirt3 also governs the detoxification of cellular ROS by regulating antioxidant enzymes [4, 29, 34, 36–38, 40]. In vitro and in vivo Sirt3-mediated deacetylation and activation of manganese superoxide dismutase (MnSOD) reduced cellular ROS levels [4, 29, 38]. Moreover, recent reports provide evidence for a Sirt3-dependent activation of the mitochondrial matrix protein isocitrate dehydrogenase 2 (IDH2), a major source of NADPH, which in turn is vital for the reduction of glutathione, an important cellular antioxidant [33, 43]. Sirt3-mediated activation of Foxo3a augmented the transcription of both MnSOD and catalase (Cat), thereby preventing mitochondrial ROS accumulation in cardiomyocytes [36]. Along these lines, Sirt3 has been reported to prevent detrimental oxidative-stress-related phenotypes in a plethora of settings, including cardiac hypertrophy, age-related hearing loss and ROS-induced embryonic developmental arrest [18, 33, 36].

Excess ROS, subsequent mitochondrial DNA damage and progressive respiratory chain dysfunction critically contribute to the development of atherosclerosis [13, 25–27]. Central risk factors of atherosclerosis such as hypercholesterolemia and hyperglycemia lead to mitochondrial dysfunction [20, 25]. Impaired mitochondrial integrity along with accumulating cellular ROS underlie vascular inflammation and promote atherogenesis from fatty streak formation over lesion progress to plaque rupture [26, 41]. In a recent study, Sirt3 deficiency and the accompanying mitochondrial protein hyperacetylation were reported to be associated with the development of the metabolic syndrome, a cluster of hallmark risk factors for atherosclerosis, including dyslipidemia, glucose intolerance, and central obesity [2, 10, 15]. To date, the role of Sirt3 in vascular biology and specifically in atherosclerosis remains unknown.

Sirt3 orchestrates mitochondrial metabolism and governs cellular ROS homeostasis in a plethora of disease-relevant settings. Thus, we hypothesized that Sirt3 provides atheroprotection and maintains metabolic homeostasis. We therefore investigated the role of Sirt3 in atherogenesis and energy expenditure using a loss-of-function approach in a mouse model of atherosclerosis.

Materials and methods

More detailed information is available in the supplemental online material.

Animals and diets

Mice were housed in cages with free access to chow and water in a temperature-controlled facility with a 12-h light/dark cycle. All experiments and animal care procedures were approved by the local veterinary authorities and carried out in accordance with our institutional guidelines. Mice with a germline *Sirt3* deletion were generated by breeding mice with floxed Sirt3 alleles ($Sirt3^{L2/L2}$) [7] with CMV-Cre deleter mice that expressed Cre in the male germline. Congenic C57BL6/J $Sirt3^{-/-}$ mice were generated through nine generations of backcrosses with C57BL6/J mice. C57BL6/J LDL receptor knockout ($Sirt3^{+/+}LDLR^{-/-}$) mice (Jackson Laboratories) were crossbred with C57BL6/J $Sirt3^{-/-}$ mice to generate C57BL6/J LDLR/Sirt3 double-knockout mice ($Sirt3^{-/-}LDLR^{-/-}$). Eight-week-old male $Sirt3^{+/+}LDLR^{-/-}$, $Sirt3^{-/-}LDLR^{-/-}$, and *wild-type* mice were fed a 1.25 % (w/w) cholesterol diet (Research Diets) or normal chow for 12 weeks and subsequently killed for fasted (unless indicated otherwise) studies.

Assessment of atherosclerosis

En face analyses of thoraco-abdominal aortae were carried out as previously described [24, 34, 42]. Briefly, thoraco-abdominal aortae were excised and opened longitudinally. Atherosclerotic plaques were visualized by fat staining using Oil red O (ORO). In addition, plaque size and composition were analyzed in serial longitudinal cryosections of aortic roots as described [34, 35]. Fibrous cap thickness and necrotic core size were assessed by Sirius Red staining. Necrotic cores were defined as areas free of extracellular matrix between the luminal fibrous cap and the collagen-rich outer intima and/or remaining media [28, 39]. Features of plaque vulnerability were characterized by necrotic core diameter and fibrous cap thickness, each of which was averaged from three different locations per mouse (once underneath each valvular leaflet) in aortic root cross sections.

Immunohistochemistry and immunofluorescence

Cryosections were blocked and stained using the following antibodies: anti-CD68, anti-CD3, and anti-vascular adhesion molecule-1 (VCAM-1; all Serotec). Collagen was visualized using Sirius Red.

Electron microscopy

Mice were killed and perfused with 1 % formaldehyde and 2 % glutaraldehyde in phosphate buffer (PB; 0.1 M; pH 7.4). Aortae were explanted and aortic wall samples (rings of 1 mm length) were extracted and postfixed by immersion in the above-described fixative for 48 h followed by three washes in PB and osmication in 1 % osmium tetroxide for 1 h. After another three washes with PB, samples were dehydrated in increasing alcohol gradients up to 100 %, embedded in Epon/Araldite resin (Sigma-Aldrich) overnight and polymerized at 60 °C during 48 h. Ultrathin sections, stained with 2 % aqueous uranyl acetate and Reynolds lead citrate were imaged using a Phillips CM 100 transmission electron microscope (FEI, Eindhoven, The Netherlands) equipped with a Gatan Orius CCD camera and Digital Micrograph acquisition software (Gatan). The cellular and subcellular structure of the inner aortic wall, specifically the endothelial monolayer and form as well as microarchitecture of endothelial mitochondria were assessed and compared in a qualitative manner. One hundred mitochondria in at least three different animals per group were assessed.

Analysis of DNA damage

Aortic DNA was extracted by ethanol precipitation using TRIzol® reagent (Sigma-Aldrich). DNA lesion frequency, halting DNA polymerase progression, was assessed using long amplicon PCR as described [9, 17]. Amplification of DNA polymerase b and b-globin were assessed as surrogate for genomic DNA lesion frequency. Amplification of a 117-bp and a 10-kb fragment of mitochondrial DNA was assessed as surrogate for mitochondrial DNA damage, which is specifically susceptible to oxidative damage. The following primers were used: b-globin 5-TTG AGA CTG TGA TTG GCA ATG CCT-3′ (sense), 5-CCT TTA ATG CCC ATC CCG GAC T-3′ (anti-sense), DNA polymerase b 5-TAT CTC TCT TCC TCT TCA CTT CTC CCC TGG-3′ (sense), 5-CGT GAT GCC GCC GTT GAG GGT CTC CTG-3′ (anti-sense), 10 kb mitochondrial fragment 5-GCC AGC CTG ACC CAT AGC CAT AAT AT-3′ (sense), 5-GAG AGA TTT TAT GGG TGT AAT GCG G-3′ (anti-sense), 117 bp mitochondrial fragment 5-CCC AGC TAC TAC CAT CAT TCA AGT-3′ (sense), 5-GAT GGT TTG GGA GAT TGG TTG ATG T-3′ (anti-sense).

Expression analyses

Aortic mRNA and protein were extracted using standard protocols. mRNA expression was analyzed by quantitative PCR using the following primers: NADP-dependent malic enzyme 5-CAG GAA CCC CCA TCT CAA C-3 (sense), 5-ACATCCTGGCTGAGGAAGC-3 (anti-sense), nicotinamide nucleotide transhydrogenase 5-GAT CCA GAT TTC CGA CTT GC-3 (sense), 5-ACT CTA CGA TGT ACT CGG CCA-3 (anti-sense), glucose-6-phosphate dehydrogenase 5-GTT GTA CCA GGG TGA TGC CT-3

(sense), 5-GCC ACC AGA TGG TAG GAT AGA-3 (anti-sense), 6-phosphogluconate dehydrogenase 5-GGGTC ATCCTGCTTGTGAAG-3 (sense), 5-CATCGATGAT GATGTCACCC-3 (anti-sense), isocitrate dehydrogenase 2 (IDH2) 5-CAG CAC TGA CTG TCC CCA G-3 (sense), 5-CAC CGT CCA TCT CCA CTA CC-3 (anti-sense), catalase (cat) 5′-CCC GCG GTC ATG ATA TTA AGT-3′ (sense), 5′-GAT GAA GCA GTG GAA GGA GC-3′ (anti-sense). Western blot analyses were performed according to standard protocols. The following specific antibodies were used: anti-SOD2 (Santa Cruz), anti-cat (Sigma).

Blood analyses

Prior to harvesting mice were fasted overnight. Blood was drawn and citrate plasma was separated from corpuscular elements by centrifugation at 4 °C immediately and stored at −80 °C until analysis. Levels of interleukin 1b (IL-1b), IL-6, monocyte chemotactic protein-1 (MCP-1), and tumor necrosis factor alpha (TNFα) were determined using Bio-Plex® multiplex array systems (Bio-Rad Laboratories).

Plasma lipoprotein fraction distribution was measured using a Roche-diagnostics Enzymatic kit for the Hitachi 902 robot according to the manufacturer's instruction.

Plasma malondialdehyde (MDA) levels were determined using the colorimetric AL detect lipid peroxidation assay (Enzo Life Sciences) according to the manufacturer's instructions. Plasma glutathione reductase activity was quantified based on the rate of NADPH oxidation using the Glutathione Reductase Assay kit (Cayman Chemical) according the manufacturer's instructions. Isocitrate dehydrogenase 2 activity was assessed based on substrate turnover using the Isocitrate Dehydrogenase Activity Colorimetric Assay kit (Biovision) according to the manufacturer's instructions. The NADP/NADPH ratio was measured using the NADP/NADPH Quantification kit (Biovision).

Glucose tolerance

For glucose tolerance tests a 200 mg/mL glucose solution was prepared and injected into the intraperitoneal cavity at 2 g/kg body weight. At regular time intervals for up to 3 h following administration, blood samples were taken and glucose was measured.

Indirect calorimetry

A 12-chamber Oxymax system (Columbus Instruments) with control of food access was used to measure oxygen consumption, carbon dioxide production, food and water intake, as well as locomotor activity in individually caged mice at 23 °C.

Mice were weighed and placed into individual metabolic cages before measurements were started. Measurements were performed for 3 days in the ad lib fed state (including 2 days of acclimatization); body weight was recorded again at the end of day 3 to rule out a loss of weight during "basal" conditions. Food access was then halted for 15 h (*Fasting*, from day 3, 5 pm through day 4, 8 am) and restored thereafter (*Refeeding*). Body weight was recorded again at the end of the experiment. Free access to water was warranted during the whole experiment. Locomotor activity (longitudinal ambulatory, "Xamb") was assessed by infrared beam interruption.

The non-protein respiratory exchange ratio, a measurement of metabolic substrate preference, was calculated as the molar ratio of $V\mathrm{co}_2$ to $V\mathrm{O}_2$. Heat (kcal/h) = $V\mathrm{O}_2$ (3.815 + 1.232 *RQ*).

Individual averaged values of metabolic data (Heat, VO_2 or respiratory quotient) for each light cycle were derived for each mouse. Group averages are presented for each genotype and light cycle.

Metabolic adaptation to *Fasting* was determined by subtracting the individual averages of *night 3* (fed) from those of *night 4* (fasted) for either heat, $V\mathrm{O}_2$ or the Respiratory Quotient. Metabolic rebound upon *refeeding* was assessed by subtracting the individual averages of *night 5* (refed) from those of *night 4* (fasted). Metabolic changes upon *fasting* and *refeeding* were also calculated as percent change.

Mitochondrial acetylation, western blotting

Mitochondria were isolated from livers and gastrocnemius muscle of mice by differential centrifugation using a Mitochondrial Isolation kit (Abcam) according to the manufacturer's instructions. Thereafter, mitochondrial protein was isolated, separated by electrophoresis and blotted using standard protocols. Membranes were probed using antibodies specific for ATPB (ATP-synthase subunit b, Abcam), acetylated lysine (AcK, Cell Signaling), and Sirt3. Antibodies recognizing murine Sirt3 were raised against the C-terminal 15-amino-acid peptide (C)DL MQR ERG KLD GQD R. The peptide was conjugated to the carrier protein KLH by the added C residue and injected into rabbits (Eurogentec). Antisera were purified using immunoaffinity chromatography.

Statistical analysis

Metric variables were assessed for distribution using Kolmogorov–Smirnov tests. Different groups were compared using unpaired Student's *t*, Mann–Whitney *U*, Kruskal–Wallis tests or two-way repeated measurements ANOVA

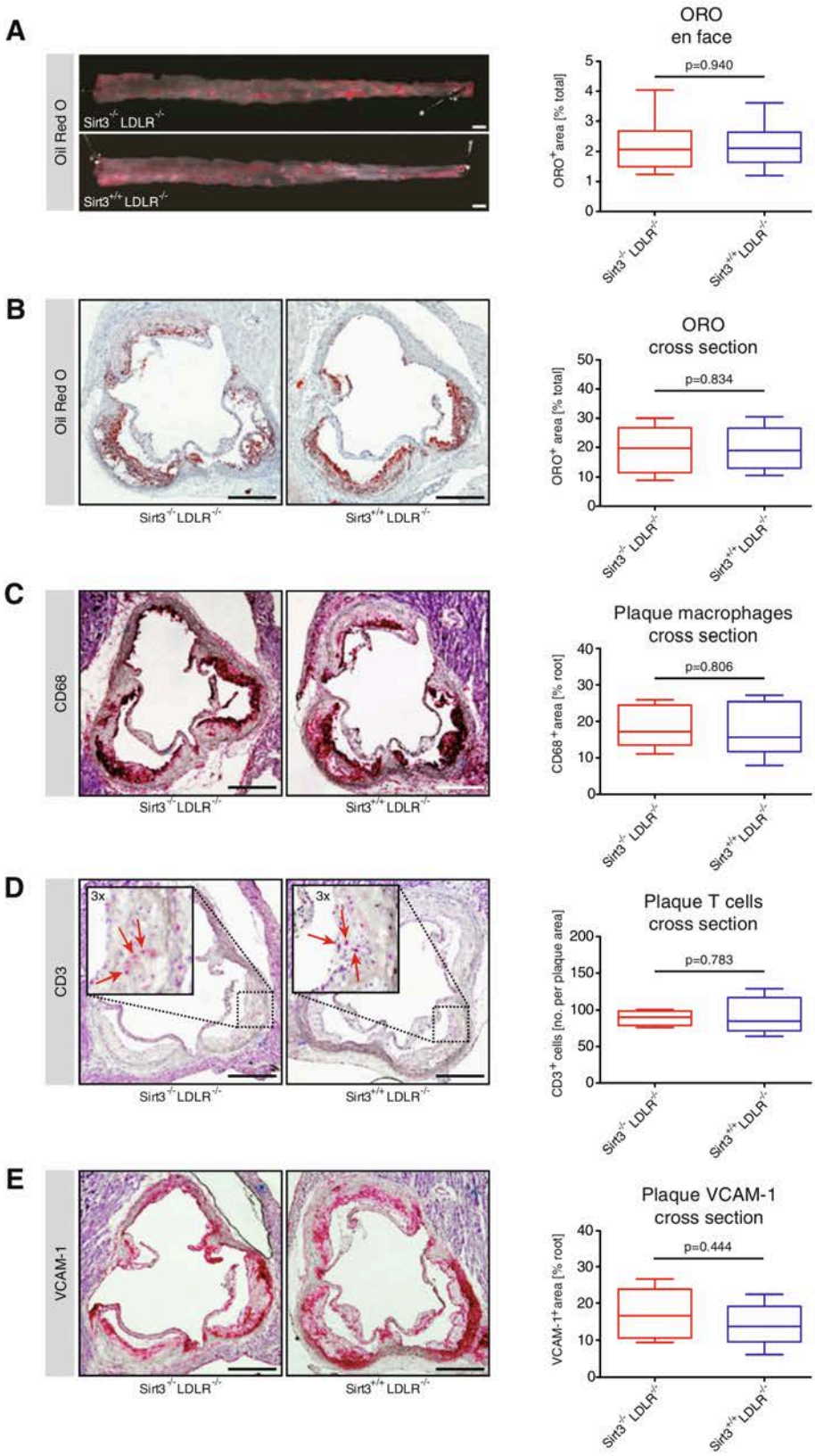

Fig. 1 Loss of Sirt3 does not affect atherosclerosis in LDLR knockout mice after a high-cholesterol diet. 8-week old male $Sirt3^{-/-}LDLR^{-/-}$ and $Sirt3^{+/+}LDLR^{-/-}$ mice were fed a high-cholesterol diet (1.25 % w/w) for 12 weeks before aortae were excised. **a** Plaque burden of thoraco-abdominal aortae en face, stained with Oil Red O (ORO), $n = 10$ per group. **b–e** Cryosections of aortic roots, $n = 6$ per group, stained with Oil red O (ORO) (**b**), or immunohistochemically for CD68 **(C),** CD3 (**d**), or vascular cell adhesion molecule-1 (VCAM-1) (**e**). Images are representative micrographs, *box plots* display interquartile ranges, *whiskers* indicate minima and maxima, *scale bars* are 1 mm (**a**) and 500 µm (**b–e**)

with Bonferroni post hoc comparisons where appropriate. Data are displayed as interquartile ranges ± minimal/maximal values, unless indicated otherwise. Null-hypotheses were rejected at $p < 0.05$, p values are two-sided. Analyses were done using Graphpad Prism version 5.0d 2010.

Results

Sirt3 deletion does not affect atherosclerosis in $LDLR^{-/-}$ mice

After 12 weeks of high-cholesterol diet, the atherosclerotic burden of $Sirt3^{-/-}LDLR^{-/-}$ and $Sirt3^{+/+}LDLR^{-/-}$ mice was assessed en face in thoraco-abdominal aortae and cross sections of aortic roots. Lesion distribution and depth, as determined by ORO stainings, did not differ between the two genotypes (Fig. 1a, b).

The cellular composition of atheromata was further assessed in adjacent cross sections of the aortic root. No differences in the immunohistochemical signals for CD68 and CD3 were observed, indicating that neither macrophage nor T cell infiltration were affected by deletion of Sirt3 in this mouse model of atherosclerosis (Fig. 1c, d). To address the degree of endothelial activation, VCAM-1 expression was investigated, again without uncovering a difference between the two groups (Fig. 1e). Complementary analyses of plasma cytokine levels addressing a potential systemic inflammatory phenotype revealed no difference in IL1b, IL6, MPC-1, and TNFα between the groups (Table 1A). Similarly, plasma total cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride levels did not differ significantly between the two groups (Table 1B).

Table 1 Deletion of Sirt3 does not affect plasma cytokine or lipid levels

	$Sirt3^{+/+}$ $LDLR^{-/-}$	$Sirt3^{-/-}$ $LDLR^{-/-}$	p value
A) Plasma cytokine levels			
IL-1b (pg/ml)	99.9 ± 17.5	128.6 ± 20.6	0.317
IL-6 (pg/ml)	2.6 ± 0.3	3.6 ± 0.3	0.073
MCP-1 (pg/ml)	34.4 ± 2.1	48.9 ± 3.8	0.052
TNFα (pg/ml)	1.2 ± 0.0	1.2 ± 0.0	0.502
B) Plasma lipid levels			
Total cholesterol (mmol/l)	30.6 ± 5.0	26.2 ± 1.6	0.421
LDL-cholesterol (mmol/l)	20.9 ± 1.8	25.4 ± 4.6	0.385
HDL-cholesterol (mmol/l)	4.7 ± 0.4	5.1 ± 0.2	0.331
Triglycerides (mmol/l)	2.60 ± 0.33	2.11 ± 0.19	0.202

Loss of Sirt3 does not alter key features of plaque vulnerability

In order to evaluate potential effects on features of plaque vulnerability, we compared fibrous cap thickness and necrotic core diameter in cross sections of the aortic roots stained for collagen. Both fibrous cap thickness and necrotic core diameter did not differ between $Sirt3^{-/-}LDLR^{-/-}$ and $Sirt3^{+/+}LDLR^{-/-}$ mice (Fig. 2a, b).

With Sirt3 orchestrating mitochondrial metabolism, we also addressed endothelial mitochondrial microarchitecture in the aortic wall using electron microscopy. A qualitative comparison of mitochondrial number and microarchitecture revealed no Sirt3-dependent difference (Fig. 3a–f).

Sirt3 deficiency is associated with increased levels of systemic but not aortic oxidative stress

To assess putative effects of Sirt3 on systemic oxidative stress in this mouse model, plasma malondialdehyde (MDA) levels were compared. Interestingly, MDA levels in $Sirt3^{-/-}LDLR^{-/-}$ mice were elevated compared with controls (Fig. 4a). Yet, a comparison of oxidative damage to the target tissue, the aortic wall, showed no Sirt3-dependent difference. Both aortic genomic and mitochondrial DNA lesion frequencies, as surrogate for oxidative DNA damage, did not differ between $Sirt3^{-/-}LDLR^{-/-}$ mice and $LDLR^{-/-}$ controls (Fig. 4b, c and Fig S1). Accordingly, aortic expression of the antioxidant enzymes superoxide dismutase 2 (SOD2) and catalase (cat) were unaffected by the lack of Sirt3 (Fig. 4d–f). Moreover, aortic expression levels of the key NADPH-producing or -regenerating enzymes, including isocitrate dehydrogenase 2 (IDH2) and malic enzyme, were unaltered between the two groups (Fig S2). Interestingly, also systemic activity levels of IDH2 and glutathione reductase (GR) as well as plasma ratios of NADP/NADPH did not differ between the two groups (Fig. 4g–i). These findings indicate that depressed glutathione regeneration was not responsible for the observed increase in systemic oxidative stress in $Sirt3^{-/-}$ mice. In absence of an antioxidative effect within the vessel wall, and given the lack of an effect of Sirt3 deletion on atherosclerosis, the increase in systemic oxidative stress appears unlikely to affect vascular inflammation and atherosclerotic burden in the context of advanced atherosclerosis.

In order to confirm a difference in Sirt3 activity in the current model ($LDLR^{-/-}$ mice on a high-cholesterol diet), we analyzed global mitochondrial protein acetylation in isolated hepatic and skeletal muscle mitochondria. Sirt3 deletion was associated with a marked mitochondrial protein hyperacetylation, suggesting a relevant difference in Sirt3 activity (Fig. 5, Fig S3C). To further exclude a diet- or LDLR-dependent blunting of Sirt3 activity, hepatic

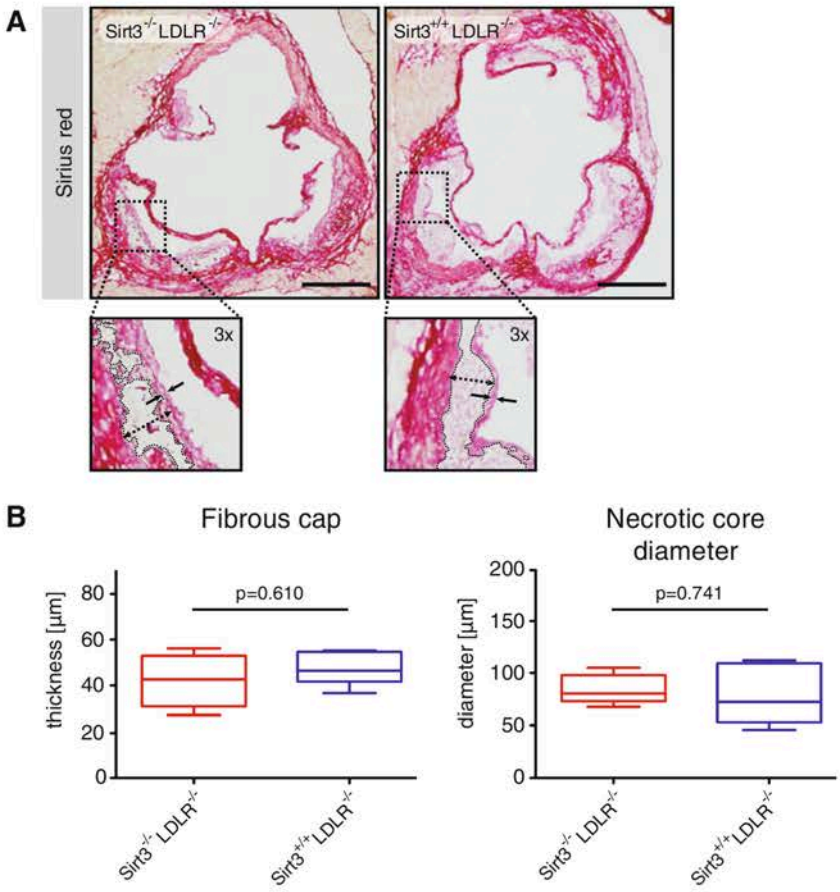

Fig. 2 Deletion of Sirt3 does not affect features of plaque stability. *Sirt3*$^{-/-}$*LDLR*$^{-/-}$ and *Sirt3*$^{+/+}$*LDLR*$^{-/-}$ mice were treated as described and aortae excised. **a** Cryosections of aortic roots, stained for collagen with Sirius red, $n = 6$ per group. **b** Quantification of fibrous cap thickness, necrotic core diameter and area. Images are representative micrographs, *box plots* display interquartile ranges, *whiskers* indicate minima and maxima, *scale bars* are 500 µm

mitochondrial acetylation was assessed in *Sirt3*$^{-/-}$*LDLR*$^{+/+}$ and *Sirt3*$^{+/+}$*LDLR*$^{+/+}$ mice fed a high-cholesterol diet or normal chow. Mitochondrial hyperacetylation in absence of Sirt3 compared with *Sirt3*$^{+/+}$ controls was similar in all settings (Fig S3).

Body weight and plasma glucose levels are increased in absence of Sirt3

Sirt3$^{-/-}$*LDLR*$^{-/-}$ mice were consistently heavier than controls (Fig. 6a). Moreover, both fed and fasted blood glucose levels were increased in absence of Sirt3 (Fig. 6b). To address whether this difference was due to a restricted capacity to metabolize glucose, we compared glucose clearance after an intraperitoneal glucose challenge. Interestingly, loss of Sirt3 did not affect glucose clearance (Fig. 6c). Moreover, plasma free fatty acid levels did not differ between *Sirt3*$^{-/-}$*LDLR*$^{-/-}$ and control mice (Fig. 6d).

In order to rule out that the difference in body weight was related to an organ-specific phenotype or organomegaly, liver, spleen and epididymal white adipose tissue weights (WAT) were assessed. No difference between the two genotypes was observed (Fig S4).

Loss of Sirt3 impairs metabolic adaptation to rapid changes in energy supply

To further investigate the metabolic phenotype of *Sirt3*$^{-/-}$*LDLR*$^{-/-}$ mice and *Sirt3*$^{+/+}$*LDLR*$^{-/-}$ controls, we compared their metabolic rates, oxygen consumption along with locomotion and food intake continuously during a period of 5 light cycles including a 15-h fasting period beginning at the end of day 3 (D3).

During the ad libitum (ad lib) period [night 1 (N1) to D3] *Sirt3*$^{-/-}$*LDLR*$^{-/-}$ mice showed a higher metabolic rate compared with controls (Fig. 7a, b left panel). The fasting-induced heat-drop was more pronounced in *Sirt3*$^{-/-}$*LDLR*$^{-/-}$ mice compared with controls (27 vs. 20 %), blunting the pre-existing difference to controls (Fig. 7a, b). Importantly, oxygen consumption (VO_2), taking individual body weight into account, did not differ between the two genotypes during the

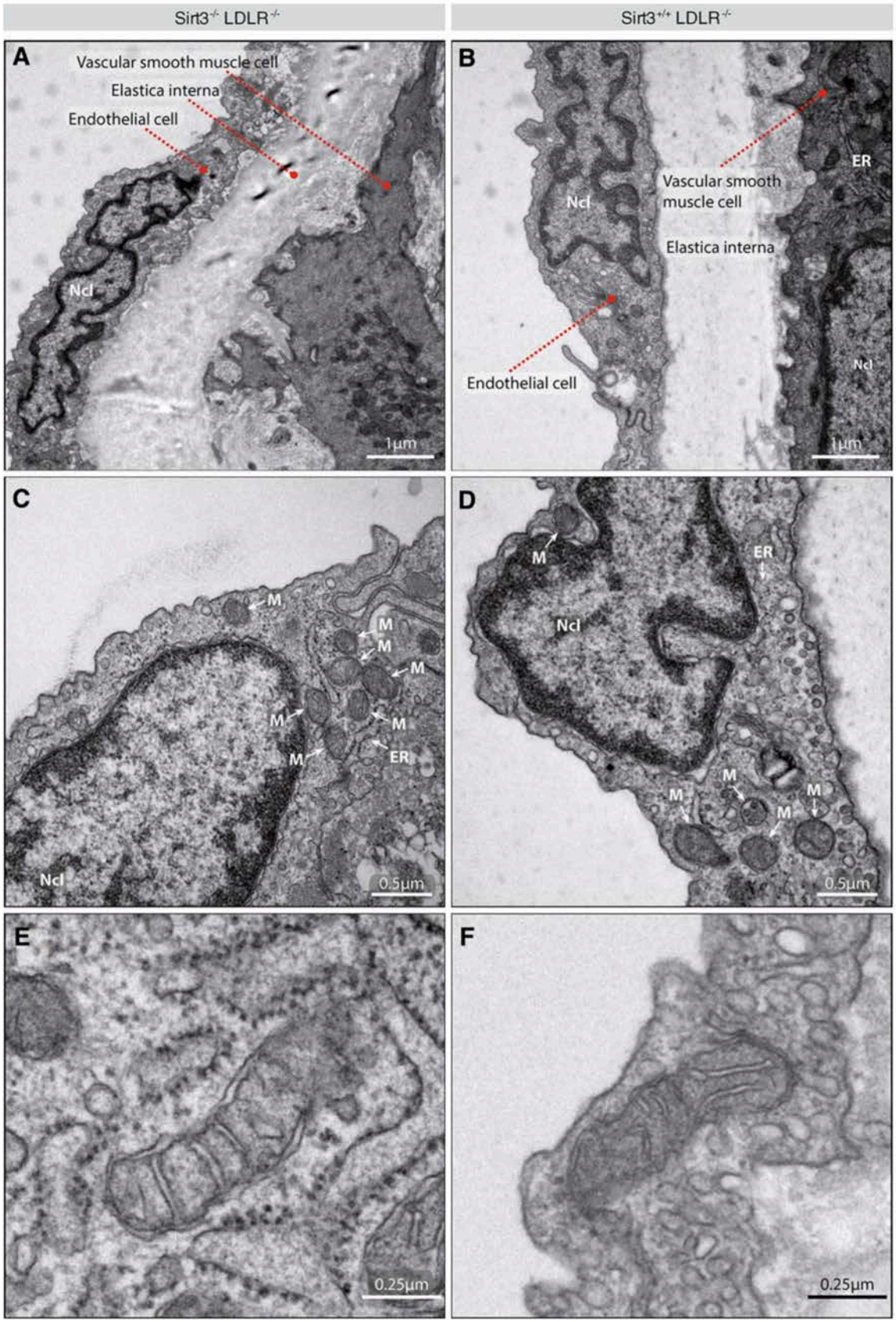
Sirt3-/- LDLR-/-
Sirt3+/+ LDLR-/-
A
Vascular smooth muscle cell
Elastica interna
Endothelial cell
Ncl
1µm
B
ER
Vascular smooth muscle cell
Ncl
Elastica interna
Endothelial cell
Ncl
1µm
C
M
M
M
M
M
M
M
ER
Ncl
0.5µm
D
M
ER
Ncl
M
M
M
M
0.5µm
E
0.25µm
F
0.25µm

Fig. 3 Sirt3 deficiency does not affect endothelial mitochondrial architecture in the aortic wall. *Sirt3*$^{-/-}$*LDLR*$^{-/-}$ and *Sirt3*$^{+/+}$*LDLR*$^{-/-}$ mice were treated as described and aortae excised and cross sections of the thoracic descending aorta were imaged using electron microscopy. **a**, **b** Overview of the inner vascular wall (from *left* to *right*): endothelial monolayer with endothelial nuclei (Ncl) protruding towards the lumen, elastica interna, vascular smooth muscle cell layer. **c**, **d** Magnification of an endothelial cell, allowing the differentiation of its subcellular components, including mitochondria (M). **e**, **f** Magnification of a single endothelial mitochondrion, showing mitochondrial microarchitecture. Images are representative micrographs of $n = 5$ per group and 100 mitochondria per aortic phenotype, and serve for qualitative comparisons only. *M* mitochondrion, *Ncl* nucleus, *ER* endoplasmic reticulum

ad lib period (Fig. 7c, left panel). However, the fasting-induced VO_2-drop was also more pronounced in *Sirt3*$^{-/-}$*LDLR*$^{-/-}$ mice compared with *Sirt3*$^{+/+}$*LDLR*$^{-/-}$ controls (24 vs. 17 %; Fig. 7c, right panels). Thus, depletion of Sirt3 was associated with an exaggerated fasting-induced hypo-metabolism compared with controls. This exaggerated hypo-metabolism upon fasting in absence of Sirt3 could not be explained by an inability to rely on lipid utilization during fasting since respiratory exchange ratios (RER) dropped to similarly low values in *Sirt3*$^{-/-}$*LDLR*$^{-/-}$ mice and *Sirt3*$^{+/+}$*LDLR*$^{-/-}$ controls (Fig S5A). Whereas cumulative food intake differed between the two groups, there was no difference when food intake was normalized to body weight (Fig S5B). The experiment itself had no effect on the body weight of the animals (Fig S5C).

During *refeeding* the metabolic rebound from the fasted state was nearly twice as pronounced in *Sirt3*$^{-/-}$*LDLR*$^{-/-}$ mice compared with controls, with heat rebound of 32 vs. 19 % in the control group and VO_2 rebound of 26 vs. 14 % in controls (Fig. 7a–c). *Refeeding* behavior in terms of body weight-adjusted food intake and locomotion did not differ between the two groups (Fig. 7d, e, Fig S5B), indicating a *metabolic* rather than a behavioral phenotype.

Discussion

Principle findings

We demonstrate for the first time that constitutive Sirt3 deletion, despite increased systemic levels of oxidative stress, neither affects the atherosclerotic burden nor features of plaque stability in *LDLR*$^{-/-}$ mice. On the other hand, Sirt3 deficiency led to a slight increase in fasting glucose levels, whereas glucose tolerance and plasma lipid levels remained unaltered. Yet, Sirt3 deletion was associated with an accelerated weight gain and an impaired capacity to cope with rapid changes in nutrient supply, showing an exaggerated fasting-induced hypo-metabolism. Similar respiratory exchange ratios rule out a Sirt3-dependent inability to rely on lipid metabolism during fasting in the applied mouse model.

Added value

To date no report on the role of Sirt3 in vascular diseases exists. With Sirt3 regulating mitochondrial oxidative metabolism and governing several ROS detoxifying systems [1, 14, 36, 38, 43], we postulated an atheroprotective role of Sirt3. Unexpectedly, Sirt3 deletion had neither relevant effects on the atherosclerotic burden nor on plaque stability. It can be speculated whether the lack of an atherosclerotic phenotype may be explained by model-associated specificities: the NAD^+-dependence of sirtuins, confining their maximal activity to times of energy deprivation, may interfere with the atherogenic high-cholesterol diet applied in this study; i.e., Sirt3 activity in the control group may have been decreased by this high-caloric diet. However, the marked mitochondrial protein hyperacetylation in *Sirt3*$^{-/-}$*LDLR*$^{-/-}$ compared with *Sirt3*$^{+/+}$*LDLR*$^{-/-}$ mice implies a relevant difference in Sirt3 activity between the groups investigated. Although currently only indirect evidence suggesting a role for Sirt3 in cellular lipid uptake exists [19, 32], an intact cholesterol uptake and metabolism system may be required for certain Sirt3-mediated effects. Thus, the absence of the LDL receptor may interfere with distinct downstream effects.

In line with previous reports that assign Sirt3 a protective role in diverse settings of oxidative damage [4, 18, 22, 33, 36], we observed increased systemic MDA levels in Sirt3-deficient hyperlipidemic mice compared with controls. In contrast to Someya et al. [32], who report on a Sirt3-dependent glutathione-mediated oxidative detoxification, glutathione reductase activity levels did not differ in the current study, suggesting other antioxidant mechanisms such as MnSOD and catalase [4, 29, 36] to be responsible for the Sirt3-mediated antioxidant protection.

Hirschey et al. [15] recently reported that Sirt3 deletion accelerated the development of the metabolic syndrome in mice when fed a high-fat diet. *Sirt3*$^{-/-}$ mice developed

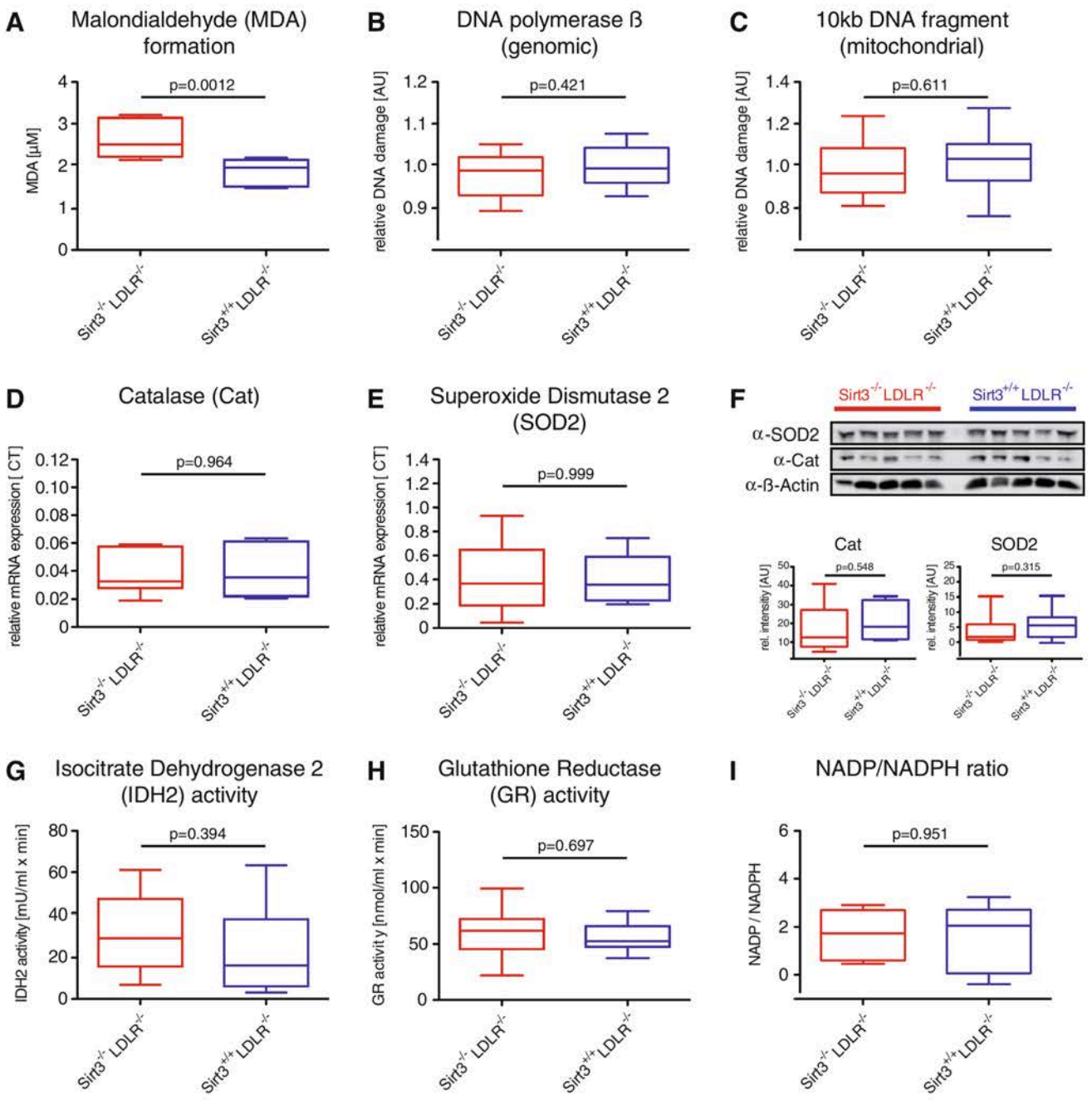

Fig. 4 Loss of Sirt3 increases systemic oxidative stress without affecting vascular oxidative DNA damage. *Sirt3*$^{-/-}$*LDLR*$^{-/-}$ and *Sirt3*$^{+/+}$*LDLR*$^{-/-}$ mice were treated as described, blood was drawn and aortas were explanted. **a** Malondialdehyde (MDA) levels as surrogate for systemic oxidative stress, $n = 8$ per group. **b** Aortic DNA was isolated and relative oxidative damage of genomic (**b**) and mitochondrial DNA **c** was assessed using quantitative PCR. **b** Lesion frequency and the resulting copy number of DNA polymerase b as surrogate for *genomic* DNA damage, $n = 10$ per group. **c** Lesion frequency and the resulting copy number of a 1 kb mitochondrial DNA fragment as surrogate for *mitochondrial* DNA damage, $n = 10$ per group. **d–f** Expression of catalase (cat) and superoxide dismutase 2 (SOD2) were assessed using quantitative PCR, $n = 9$ per group (**d**, **e**) and by western blot, $n = 5$ per group (**f**). **g** Plasma isocitrate dehydrogenase 2 (IDH2) activity, $n = 9$ per group. **h** Plasma glutathione reductase (GR) activity, $n = 6$ per group. **i** Plasma NADP/NADPH ratio, $n = 5$ per group. *Box plots* display interquartile ranges, *whiskers* indicate minima and maxima

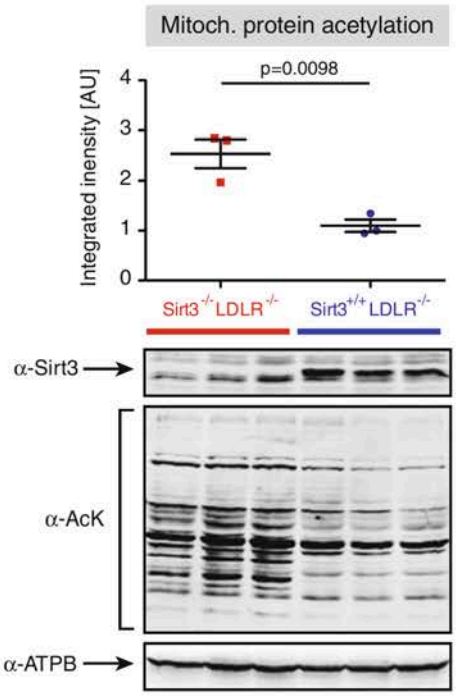

Fig. 5 Loss of Sirt3 lead to global mitochondrial hyperacetylation. Hepatic mitochondria were isolated and protein was extracted, separated by electrophoresis and probed for Sirt3 (α-Sirt3), acetylated lysine residues (α-AcK) and the beta-subunit of ATP-Synthase (α-ATPB, loading control). Data are mean ± SEMs with superimposition of individual data points

accelerated obesity, insulin resistance, and hyperlipidemia. In parallel, loss of Sirt3 was associated with accelerated weight gain and elevated plasma glucose in *LDLR*$^{-/-}$ mice in the current study; however, without affecting serum lipid levels or glucose tolerance, thus not fulfilling the criteria for a full-grown metabolic syndrome [2]. Detailed assessment of this metabolic phenotype showed an impaired capacity of *Sirt3*-deficient mice to cope with rapid changes in nutrient supply. Although driving not only glucose and amino acid catabolism, but also fatty acid oxidation in times of energy deprivation [14], similar respiratory exchange ratios both during fed ad libitum and fasting periods rule out an impaired fatty acid utilization in absence of Sirt3. Interestingly, neither muscle- nor liver-specific loss of Sirt3 did manifest any metabolic phenotype under either chow or high-fat diet [7], indicating that either another organ or the interplay between different organs are necessary for Sirt3 to exert its metabolic functions in its entirety.

Potential limitations

This study has to be interpreted in light of the following limitations: experiments have been carried out in an *LDLR*$^{-/-}$ model using a high-cholesterol diet. Therefore, a potential interplay between Sirt3 and the LDL receptor in wild-type mice under similar conditions cannot be excluded. However, to date no evidence for an LDLR-mediated role of Sirt3 exists. Moreover, a putative effect of Sirt3 on early atherosclerosis or vascular function was not assessed. Given the increased MDA levels in *Sirt3*-deficient mice Sirt3 may affect endothelial relaxation, thereby affecting the initial disposition for atherogenesis. In addition, a diet-induced blunting of Sirt3 activity cannot be ruled out completely. However, a persistent mitochondrial hyperacetylation in *Sirt3*-deficient mice, irrespective of the diet supplied or LDLR expression strongly suggests no relevant diet- or LDLR-dependent blunting of Sirt3 activity. Moreover, the electron microscopy-based analyses of the inner aortic wall and endothelial subcellular structures are qualitative in nature; no quantitative statements can be made. Finally, the exact mechanisms underlying the constrained ability of *Sirt3*-deficient mice to cope with rapid changes in nutrient supply and the causes of the accelerated weight gain remain to be determined.

Implications

The current study provides a first step in unraveling the role of Sirt3, a key enzyme in metabolic regulation and ROS homeostasis, in vascular disease. Surprisingly, no effects on advanced atherosclerotic lesions were observed, even though levels of systemic oxidative stress were increased in absence of Sirt3. A striking acceleration in weight gain and an impaired capacity to react to rapid changes in nutrient supply underline the importance of Sirt3 in energy homeostasis. The latter findings assign Sirt3 a potential role in the development of cardiovascular risk factors, postponing the onset of distinct metabolic risk factors.

Further studies will be needed to determine the role of Sirt3 on vascular function; a reassessment of putative effects on atherosclerosis using gain-of-function studies, in the absence of a high-caloric diet, and an extended analysis of the metabolic phenotype of Sirt3 on a wild-type background and chow diet will shed further light on the putative protective roles of Sirt3 in vascular health and disease.

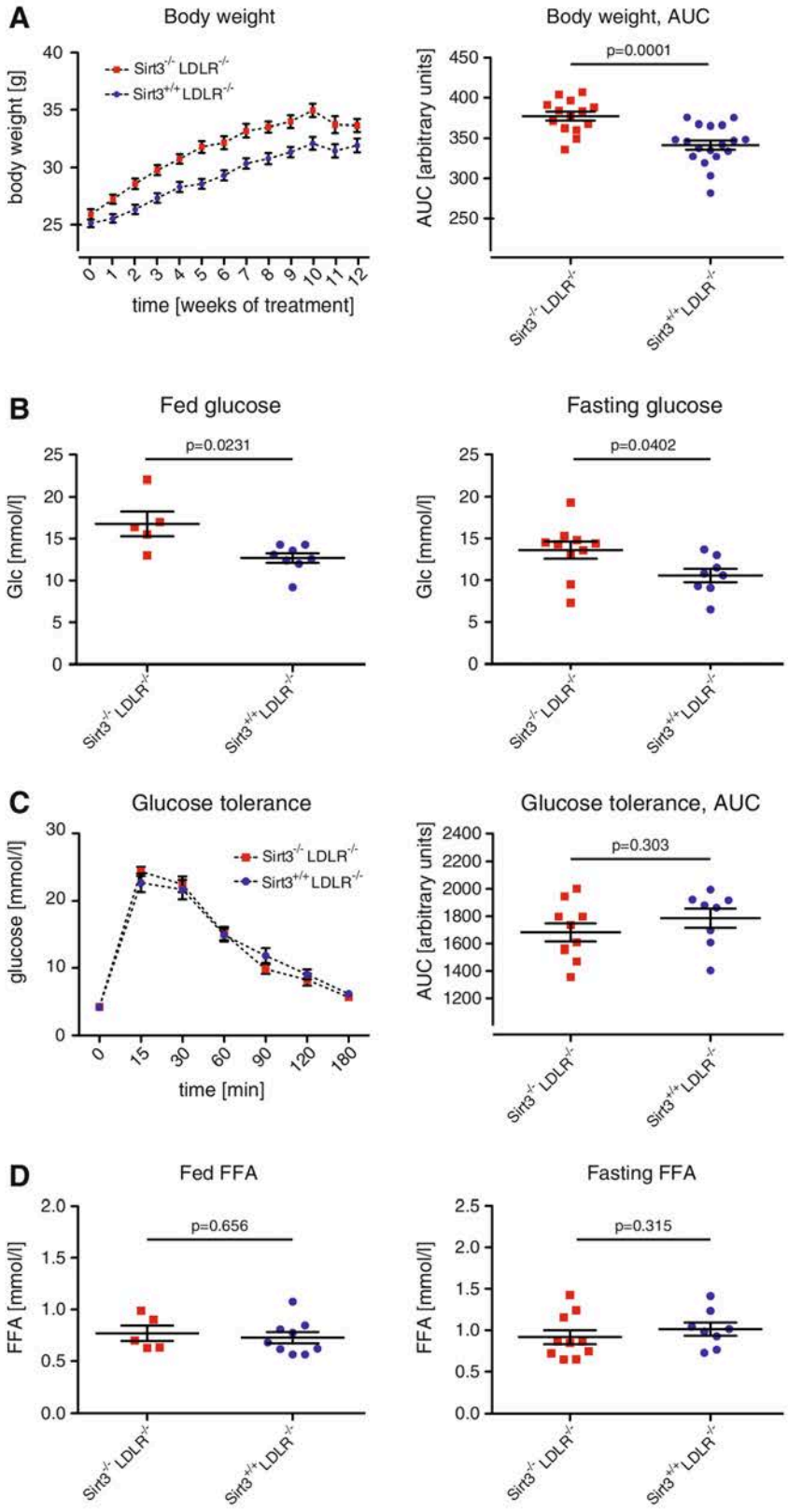

Fig. 6 Deletion of Sirt3 accelerates weight gain and increases plasma glucose levels. *Sirt3*$^{-/-}$*LDLR*$^{-/-}$ and *Sirt3*$^{+/+}$ *LDLR*$^{-/-}$ mice were fed a high-cholesterol diet (1.25 % w/w) for 12 weeks. **a** Weight gain during treatment (*left panel*) and its quantification comparing areas under the curve (AUC, *right panel*). **b** Plasma glucose levels, fed (*left panel*) and fasted (*right panel*). **c** Plasma glucose levels upon intraperitoneal glucose challenge (2 g/kg body weight) (*left panel*), quantification comparing areas under the curve (AUC, *right panel*). **d** Plasma free fatty acid (FFA) content, fed (*left panel*) and fasted (*right panel*). Data are means ± SEMs with superimposition of individual data points in all panels except for **a** and **c**, *left panels*

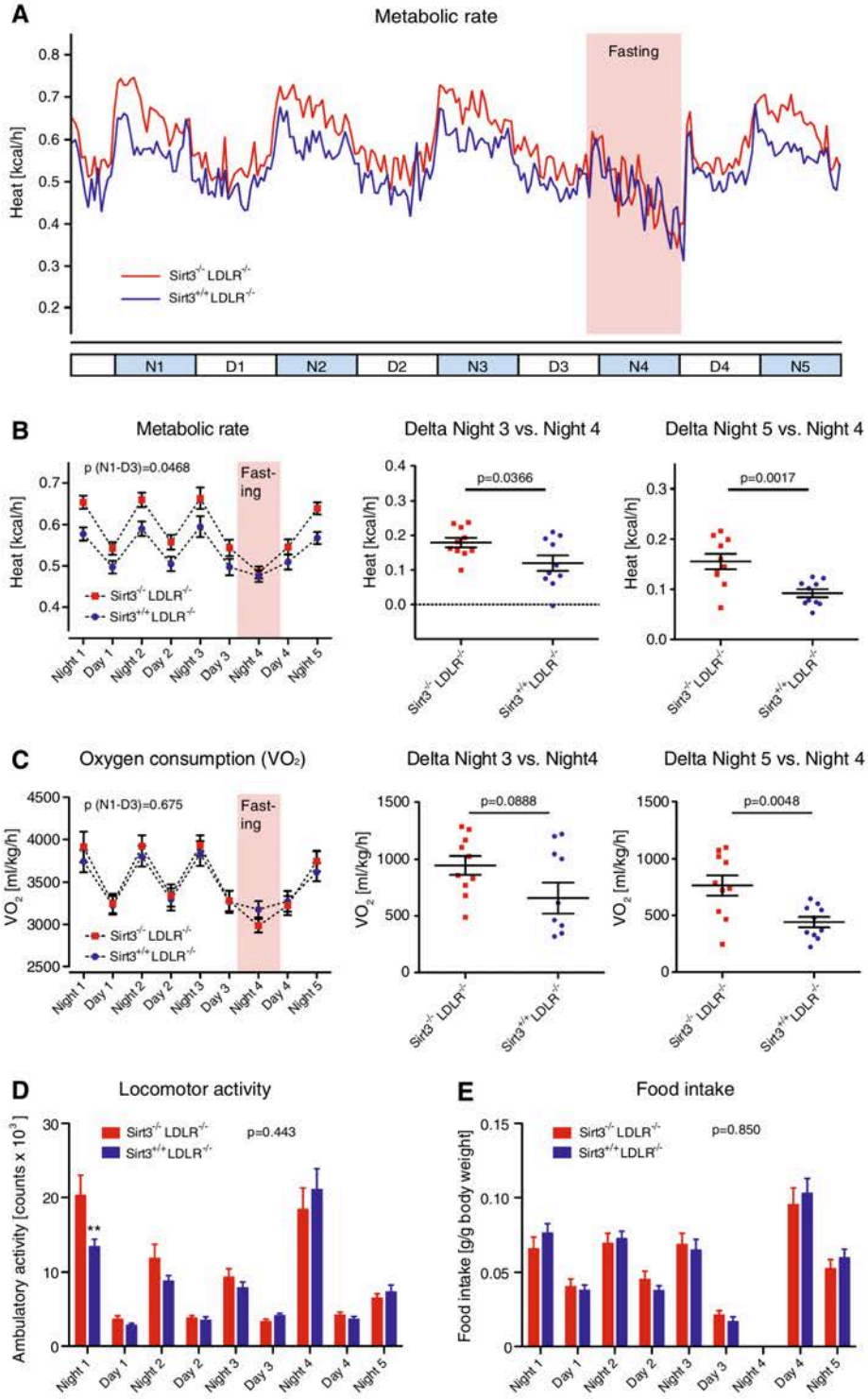

Fig. 7 Loss of Sirt3 impairs metabolic adaptation to rapid changes in energy supply. After a 12-week high-cholesterol diet (1.25 % w/w) different metabolic parameters were assessed in individually caged *Sirt3*$^{-/-}$*LDLR*$^{-/-}$ and *Sirt3*$^{+/+}$*LDLR*$^{-/-}$ mice during five light cycles. **a**, **b** Metabolic rate (heat production): circadian profile during an ad libitum fed state (night 1 to day 3), during a 15-h overnight fasting period (5 pm, day 3 through 8 am, day 4) and during subsequent refeeding (day 4, night 5), *N* night, *D* day. **b** Average metabolic rates per day/night (*left panel*); metabolic drop during fasting ("delta night 3 vs. night 4", *center panel*), and metabolic rebound upon refeeding ("delta night 4 vs. night 5", *right panel*). **c** Average oxygen consumption (VO_2) per day/night (*left panel*); VO_2 drop during fasting ("delta night 3 vs. night 4", *center panel*), and VO_2 rebound upon refeeding ("delta night 4 vs. night 5", right panel). **d** Average locomotor activity per day/night. **e** Average food intake per day/night. Data are means ± SEM, with superimposition of individual data points in "delta" *panels*. *) $p < 0.01$ compared with *LDLR*$^{-/-}$*Sirt3*$^{-/-}$ mice

Acknowledgments We are grateful to Marianne Carrard for technical assistance in biochemical analyses and to Therese Bruggmann for assistance in electron microscopy. This study was funded by the Swiss National Science Foundation, Bern, Switzerland (310030-130626/1 to C.M.M. and 310030-118353 to T.F.L.), the European Research Council (ERC-AdG-231138-sirtuins to J.A.), the Swiss Heart Foundation, Bern, Switzerland (C.M.M.), Matching Funds, University of Zurich, Switzerland (C.M.M., S.W., T.F.L.), and the Foundation for Cardiovascular Research, Zurich, Switzerland.

Conflict of interest None.

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Deletion of Sirt3 does not affect atherosclerosis but accelerates weight gain and impairs rapid metabolic adaptation in LDL receptor knockout mice – Implications for cardiovascular risk factor development

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Running title: Sirt3 in Atherosclerosis & Metabolism

Key words: Sirtuins; Sirtuin 3; atherosclerosis; metabolism; oxidative stress

SUPPLEMENTAL MATERIAL

Figure S1: Loss of Sirt3 does not increase aortic oxidative DNA damage.

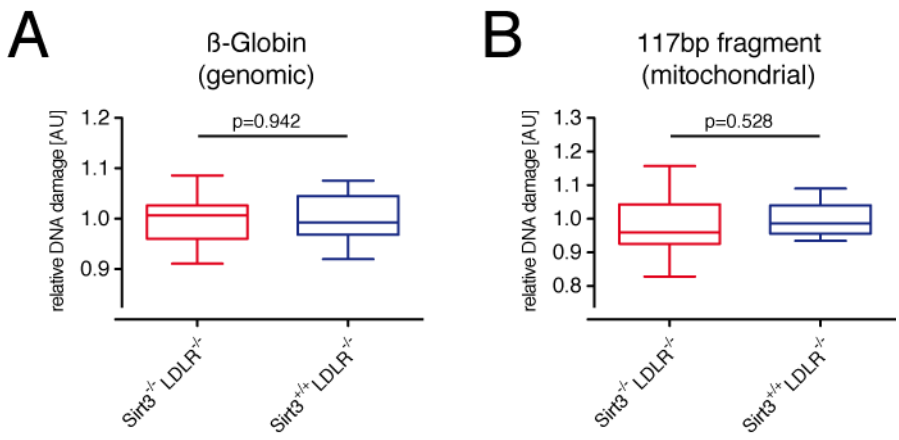

Figure S1: Eight-week old male *LDLR*$^{-/-}$ and *LDLR*$^{-/-}$*Sirt3*$^{-/-}$ mice were fed a high-cholesterol diet (1.25% w/w) for 12 weeks before aortae were harvested. Aortic DNA was isolated and relative oxidative damage of genomic (A) and mitochondrial DNA **(**B) was assessed using quantitative PCR. **(A)** Lesion frequency and the resulting copy number of ß-Globin served as surrogate for *genomic* DNA damage. **(B)** Lesion frequency and the resulting copy number of a 117bp mitochondrial DNA fragment served as surrogate for *mitochondrial* DNA damage. Box plots show interquartile ranges, whiskers indicate minima and maxima.

Figure S2: Loss of Sirt3 does not affect aortic expression levels of major NADPH regenerating enzymes.

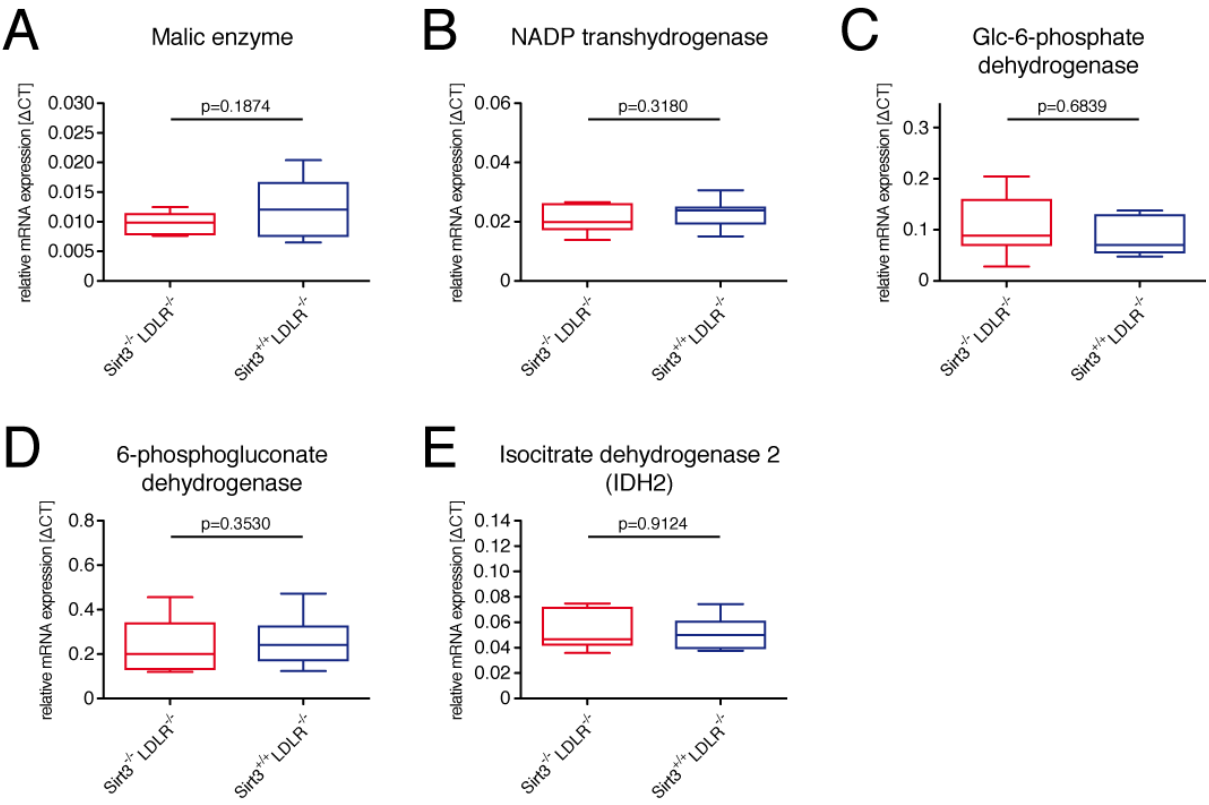

Figure S2: Eight-week old male *LDLR*$^{-/-}$ and *LDLR*$^{-/-}$ *Sirt3*$^{-/-}$ mice were fed a high-cholesterol diet (1.25% w/w) for 12 weeks before mice were harvested and mRNA was isolated. Aortic expression analyses of the key NADPH regenerating enzymes were assessed using quantitative PCR. **(A)** Malic enzyme. **(B)** NADPH transhydrogenase. **(C)** Glucose-6-phosphate dehydrogenase (Glc-6-phosphate dehydrogenase). **(D)** 6-Phosphogluconate dehydrogenase. **(E)** Isocitrate dehydrogenase 2 (IDH2). Box plots show interquartile ranges, whiskers indicate minima and maxima.

Figure S3: Sirt3 deficiency leads to hepatic global mitochondrial hyperacetylation both after high-cholesterol diet and normal chow.

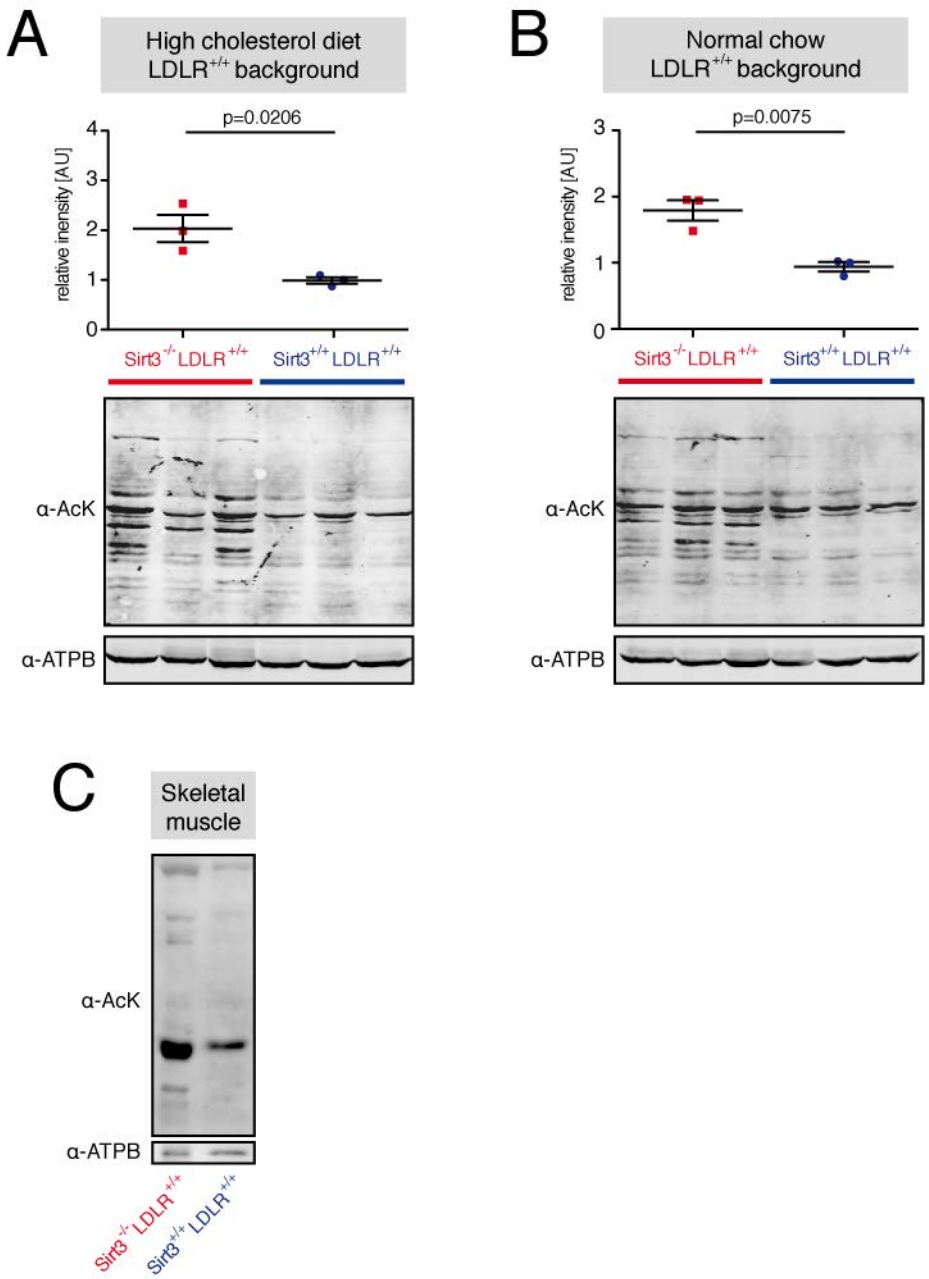

Figure S3: Eight-week old male *Sirt3*$^{-/-}$, *Sirt3*$^{-/-}$ *LDLR*$^{-/-}$, and wiltdtype mice, respectively, were fed a high-cholesterol diet (1.25% w/w) or normal chow for 12 weeks before mice were harvested. Mitochondrial protein was isolated from livers (A & B) and gastrocnemius muscle, respectively, electrophoretically separated and probed with anti-acetyl lysine (α-AcK). **(A)** Hepatic mitochondrial protein acetylation after 12 weeks of high-cholesterol diet. **(B)** Hepatic mitochondrial protein acetylation after 12 weeks of normal chow. **(C)** Gastrocnemic mitochondrial protein acetylation after 12 weeks of high-cholesterol diet. ATP-synthase subunit ß (ATPB) served as loading control. Data are presented as means ± SEM with superimposition of individual data points.

Figure S4: Loss of Sirt3 does not affect epididymal white adipose tissue, liver or spleen mass in *LDLR*$^{-/-}$ mice.

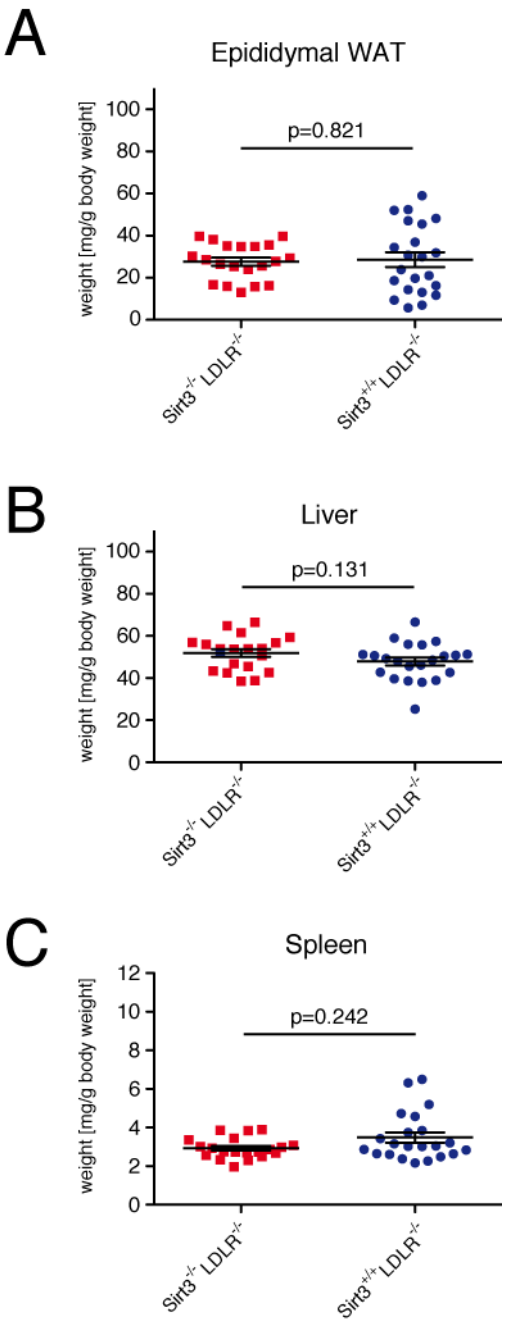

Figure S4: Eight-week old male *LDLR*$^{-/-}$ and *LDLR*$^{-/-}$*Sirt3*$^{-/-}$ mice were fed a high-cholesterol diet (1.25% w/w) for 12 weeks before mice were harvested. **(A)** Epididymal white adipose tissue (WAT) mass. **(B)** Liver mass. **(C)** Spleen mass. Data are presented as means ± SEM with superimposition of individual data points.

Figure S5: **Loss of Sirt3 does not affect metabolic substrate preference or food intake.**

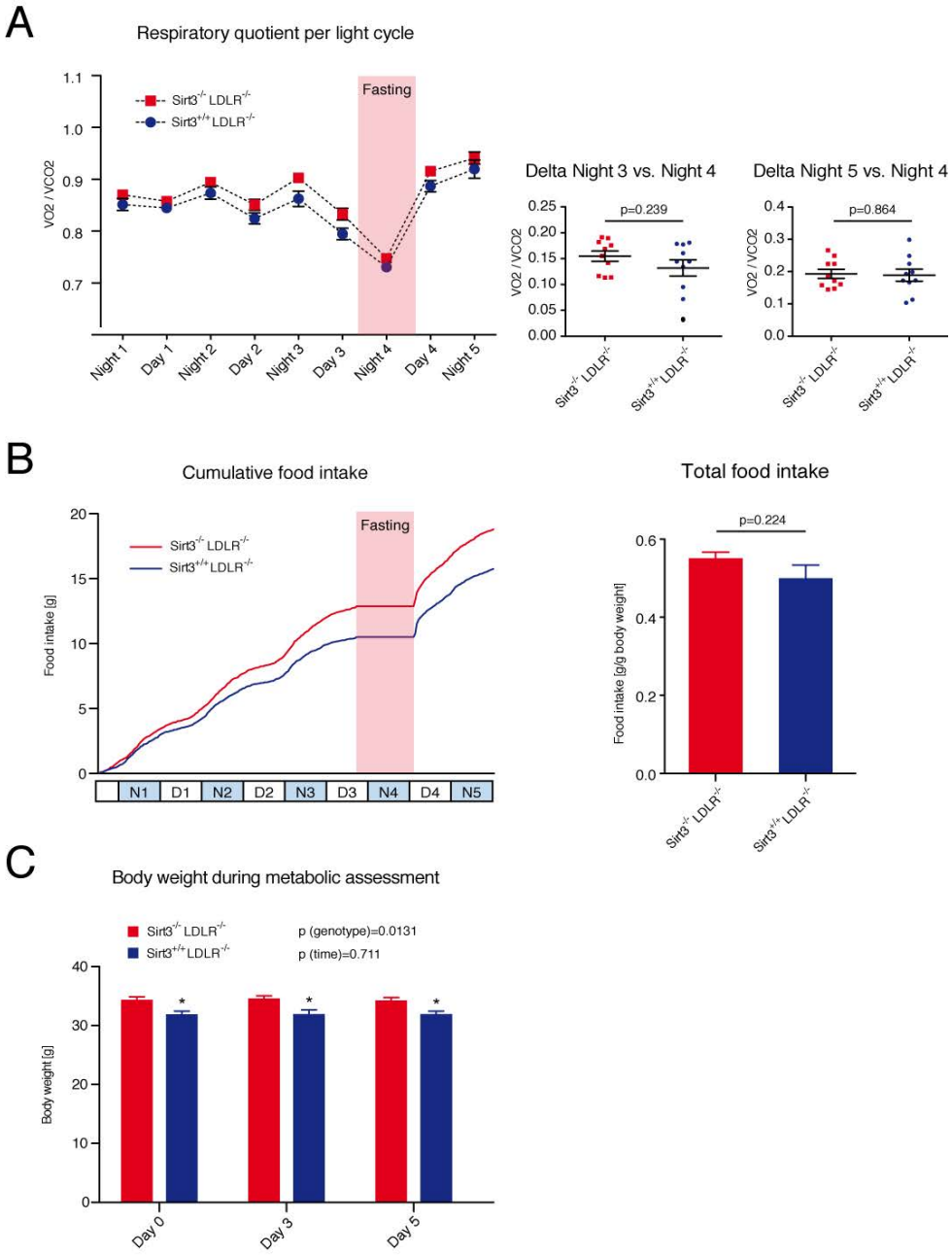

Figure S5: After a 12-week high-cholesterol diet (1.25% w/w) different metabolic parameters were assessed in individually-caged *LDLR*$^{-/-}$ and *LDLR*$^{-/-}$*Sirt3*$^{-/-}$ mice during five light cycles. **(A)** Respiratory quotient averaged per day/night (left panel); respiratory quotient drop during fasting, determined by subtracting the individual, fed (Night 3) to fasted (Night 4) averages (center panel, « Delta N3 vs. N4 ») and respiratory quotient rebound upon refeeding, determined by subtracting refed (Night 5) to fasted (Night 4) averages (right panel, « Delta N4 vs. N5 »). **(B)** Cumulative, real-time feeding (left panel) and total feeding (right panel) over the whole experiment. **(C)** Body weights before (Day 0), during (Day 3) and after (Day 5) the experiment. Data are presented as means ± SEM, with superimposition of individual data points in « Delta » panels. N=Night, D=Day. *) $p<0.05$ compared with *LDLR*$^{-/-}$ *Sirt3*$^{-/-}$ mice.

SUPPLEMENTARY METHODS

Tissue harvesting and processing

Mice were anesthetized using isoflurane. After medial thoraco- and laparotomy the left ventricle was punctured and blood was drawn. Thereafter, the right atrium was incised and the vascular system was rinsed briefly with cold normal saline (0.9% w/v) before organs were explanted. For histological examination, tissue was embedded in OCT (optimal cutting temperature) compound (Tissue-Tek) and immediately frozen on dry ice; for biochemical analyses samples were snap frozen in liquid nitrogen. All samples were stored at -80°C until analysis.

6.2 Mild endothelial dysfunction in Sirt3 knockout mice fed a high-cholesterol diet: protective role of a novel C/EBP-β-dependent feedback regulation of SOD2

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Status of the manuscript:

Published in:	Basic Research in Cardiology 2016 May;111(3):33.
Published online:	12 April 2016
DOI:	10.1007/s00395-016-0552-7
PMID:	27071400
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Author contributions Daniel S. Gaul:

- Planning, data acquisition, analysis, statistical evaluation, and interpretation of experiments
- Contributions to the individual figures:
    - Figure 1: contributed subfigures 1A and C
    - Figures 3 and 4: contributed the whole figures
    - Supplemental Figures 3 and 4 (S3 and S4): contributed the whole figure S3 and subfigures S4A-S4D
    - Supervision of visiting PhD student Lisa Pasterk and Master student Natacha Calatayud, who, in collaboration with DSG contributed Figure 5, S4E and S5.
- Editing and proofreading of the manuscript

Basic Res Cardiol (2016) 111:33
DOI 10.1007/s00395-016-0552-7

ORIGINAL CONTRIBUTION

Mild endothelial dysfunction in Sirt3 knockout mice fed a high-cholesterol diet: protective role of a novel C/EBP-β-dependent feedback regulation of SOD2

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Received: 22 July 2015 / Accepted: 30 March 2016 / Published online: 12 April 2016

Abstract Sirtuin 3 (Sirt3) is an NAD^+-dependent mitochondrial deacetylase associated with superoxide dismutase 2 (SOD2)-mediated protection from oxidative stress. We have reported accelerated weight gain and impaired metabolic flexibility in atherosclerotic *Sirt3*$^{-/-}$ mice. Oxidative stress is a hallmark of endothelial dysfunction. Yet, the role of Sirt3 in this context remains unknown. Thus, we aimed to unravel the effects of endogenous Sirt3 on endothelial function and oxidative stress. Knockdown of Sirt3 in human aortic endothelial cells (HAEC) increased intracellular mitochondrial superoxide accumulation, as assessed by electron spin resonance spectroscopy and fluorescence imaging. Endothelium-dependent relaxation of aortic rings from *Sirt3*$^{-/-}$ mice exposed to a normal diet did not differ from *wild-type* controls. However, following 12 weeks of high-cholesterol diet and increasing oxidative stress, endothelial function of *Sirt3*$^{-/-}$ mice was mildly impaired compared with *wild-type* controls. Relaxation was restored upon enhanced superoxide scavenging using pegylated superoxide dismutase. Knockdown of *Sirt3* in cultured HAEC diminished SOD2 specific activity, which was compensated for by a CCAAT/enhancer binding protein beta (C/EBP-β)-dependent transcriptional induction of SOD2. Abrogation of this feedback regulation by simultaneous knockdown of *C/EBP-β* and *Sirt3* exacerbated mitochondrial superoxide accumulation and culminated into endothelial cell death upon prolonged culture. Taken together, *Sirt3* deficiency induces a mild, superoxide-dependent endothelial dysfunction in mice fed a high-cholesterol diet. In cultured endothelial cells, a novel C/EBP-β-dependent rescue mechanism maintains net SOD2 activity upon transient knockdown of *Sirt3*.

Keywords Sirt3 · Oxidative stress · SOD2 · C/EBP-β · Endothelial function

S. Winnik and D. S. Gaul contributed equally.

Electronic supplementary material The online version of this article (doi:10.1007/s00395-016-0552-7) contains supplementary material, which is available to authorized users.

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Introduction

Sirtuin 3 (Sirt3) is a mitochondrial deacetylase that regulates mitochondrial oxidative metabolism and detoxification from oxygen radicals [1, 3, 44, 58]. It is the third out of seven mammalian homologues of the so-called class III histone deacetylases (HDACs). They share a conserved catalytic binding motif for the oxidized form of nicotinamide adenine dinucleotide (NAD^+), defining them as

class III HDACs and concentrating their activity to times of low cellular energy levels [7, 9, 14, 19, 58]. Since its first description in 1935, caloric restriction is the only intervention that has been shown to prolong lifespan and maintain mammalian health [25, 32, 47]. Mammalian sirtuins provide protective effects in a variety of age-related pathologies, thus promoting healthy aging [7, 13, 23, 34]. Sirt3 is one out of two sirtuins that have been associated with human longevity and health span [4, 15, 46]. Cardiovascular diseases, including myocardial infarction and stroke, are the leading global cause for age-related morbidity and mortality, accounting for 17.3 million deaths a year, with an estimated rise to >23.6 million by 2030 [2, 26].

Recently, Sirt3 deficiency has been reported to accelerate the development of the metabolic syndrome, a cluster of hallmark risk factors for cardiovascular disease [18]. Although we did not observe an atherosclerotic phenotype in low-density lipoprotein receptor knockout mice lacking *Sirt3*, its loss was associated with accelerated weight gain, impaired metabolic adaptation and increased levels of systemic oxidative stress [55].

Excess endothelial reactive oxygen species (ROS), subsequent mitochondrial DNA damage and progressive respiratory chain dysfunction are essential in the development of endothelial dysfunction, an early event in and an independent predictor of cardiovascular disease [17, 28, 29, 33, 36, 40, 41, 48]. In numerous settings including embryonic development, age-related hearing loss, neuronal injury, and cardiac hypertrophy, Sirt3 has been shown to protect from oxidative stress [10, 22, 49, 51]. In the majority of settings, Sirt3 augmented superoxide scavenging by enhancing superoxide dismutase 2 (SOD2) and/or catalase (cat) activity [42, 51, 53]. Whether increased SOD2 activity is mediated by direct deacetylation or transcriptional regulation remains controversial [51, 53]. Moreover, Sirt3 activates isocitrate dehydrogenase 2 (IDH2), which drives the tricarboxylic acid (TCA) cycle and is a major donor of NADPH, an essential cofactor for both glutathione regeneration and endothelial nitric oxide synthase (eNOS)-mediated NO formation [31, 43, 44, 59]. Hitherto, only few data exist on the role of Sirt3 in *arterial* endothelial cells [35]. No data are available on its effects on endothelium-dependent vasodilation.

In contrast to the constitutive expression of the cytosolic and extracellular isoforms SOD1 and SOD3, the inducible expression of SOD2 allows the response to varying levels of intracellular oxidative stress. The acetylation-dependent transcription factor CCAAT/enhancer binding protein beta (C/EBP-β) has been shown to bind an intronic TNF-responsive element of SOD2 and thereby facilitate SOD2 gene transcription in response to various stimuli associated with increased levels of intracellular ROS [6, 21, 30].

Our goal was to unravel the effects of endothelial Sirt3 on oxidative stress and endothelial function, and to investigate the underlying mechanisms in human aortic endothelial cells. To enhance oxidative stress in aortae, we exposed mice to a high-cholesterol diet [37]. In the current work, we uncovered a C/EBP-β-dependent induction of SOD2 expression as rescue mechanism for the Sirt3-dependent loss of SOD2 activity, an interaction, that until to date remained unknown.

Methods

Mice

Mice were housed in a temperature-controlled facility with a 12-h light/dark cycle and free access to chow and water. All animal studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Mice with a germline *Sirt3* deletion were generated as described. [12, 55] Congenic C57BL6/J $Sirt3^{-/-}$ mice were generated through nine generations of backcrosses with C57BL6/J mice. Eight-week-old male $Sirt3^{-/-}$ and *wild-type* mice were fed a 1.25 % (w/w) cholesterol diet (research diets) for 12 weeks and subsequently killed for fasted (unless indicated otherwise) studies.

Endothelial function

Endothelium-dependent vasorelaxation was investigated as described [37, 56]. Briefly, aortae were explanted and aortic rings were obtained. Relaxation in response to acetylcholine (ACh) or sodium nitroprusside (SNP) was assessed using isometric force transducers in organ chamber baths (Multimyograph, DMT). Maximal contraction was defined before initiating the experiment using potassium chloride (KCl) in a concentration of 80 mM. Precontraction to a maximum of 70 % maximal contraction was achieved using norepinephrine (NE) in a dose of 10^{-7} M. Dose–response curves were quantified comparing areas under the curves (AUC).

Cell culture and transfection

Human aortic endothelial cells (HAEC, Cambrex) from passage three to eight were grown to confluence at 5 % CO_2 and 37 °C in Endothelial Growth Medium 2 (Lonza) supplemented with 10 % fetal calf serum. Transient knockdown was performed using Lipofectamine® Reagent (Life Technologies) for transfection of the following small interference RNA (siRNA): Sirt3 (5′-GCC CAA CGU

CAC UCA CUA CUU TT-3′), C/EBP-β (Trilencer-27 siRNA, OriGene), SOD2 (5′-AAU GCU ACA AUA GAG CAG CUU TT-3′), scrambled (5′-UUC UCC GAA CGU GGC ACG ATT-3′), Trilencer-27 Universal Scrambled Negative Control siRNA (SR30004, Origene), and Silencer Negative Control #5 siRNA (AM4642, Ambion). Total siRNA amounts were kept equal among all experiments. Where two-stage transfections (double-knockdown of *Sirt3* and *C/EBP-β*) were performed, all groups in the respective experiments were transfected twice. Knockdown efficiency was assessed using expression analyses on RNA- (quantitative PCR) and protein level (western blot).

Expression analyses

RNA isolation, reverse transcription and SYBR® green-based (Applied Biosystems) quantitative PCR was carried according to standard protocols using a Quant Studio 7 Flex Real Time PCR thermocycler (Applied Biosystems) with the associated sequence detection system and software. Expression was calculated using the $\Delta\Delta C_T$ method. Relative gene expression was normalized to β-actin (house-keeping gene). Western blot analyses of HAEC lysates were conducted according to standard protocols using the following specific antibodies:

anti-Sirt3 (rabbit monoclonal, Cell Signalling Technology), anti-C/EBP-β [C19] (rabbit polyclonal, Santa Cruz Biotechnology), anti-SOD2 (rabbit polyclonal, Abcam), anti-catalase (mouse monoclonal, Sigma), anti-glutathion peroxidase 1 (rabbit polyclonal, Novus Biologicals), anti-total eNOS (mouse polyclonal, BD Transduction Laboratories), anti-eNOS (pThr495) (mouse, monoclonal, BD Transduction Laboratories), anti-eNOS (pSer1177) (mouse, monoclonal, BD Transduction laboratories), anti-xanthine oxidase (rabbit polyclonal, Biorbit), anti-NADPH oxidase subunit $p22^{phox}$ (rabbit polyclonal, Biorbit), and anti-β actin (mouse monoclonal, Sigma-Aldrich). Specific signals were detected using species-specific secondary antibodies.

Immunoprecipitation

HAEC were cultured in 10 cm cell culture dishes, transfected as described above and lysed in 1 ml radioimmunoprecipitation assay (RIPA) buffer. Samples were kept on ice throughout IP steps. The lysates were pre-cleared with 30 µl washed Protein G Agarose beads (Millipore) and, after removal of the beads, incubated over night with suitable monoclonal antibodies for SOD2, C/EBP-β or Sp1, respectively. 30 µl of washed Protein G Agarose beads were added and the mixture was incubated for 4.5 h with agitation on the incubation wheel. Beads–antibody–antigen complexes were separated from the lysates by centrifugation and the pellet washed three times with RIPA buffer. After adding 30 µl of 4× Laemmli buffer, the samples were incubated at 60 °C with shaking for 10 min and the supernatant resulting from subsequent centrifugation was analyzed by western blotting.

The following antibodies were used for immunoprecipitation and subsequent determination of the acetylation or nitrosylation status, respectively: anti-SOD2 [1E8] (mouse monoclonal, Abnova), anti-acetyl lysine (rabbit polyclonal, Chemicon), and anti-nitro tyrosine [HM.11] (mouse monoclonal, Abcam).

Electron spin resonance spectroscopy

Intracellular superoxide in HAEC was detected by electron spin resonance (ESR) spectroscopy using the superoxide-specific spin trap 1-hydroxy-3-methoxy-2,2,5,5-tetramethylpyorrolidine (CMH, Noxygen) as described [50, 57].

Mitochondrial superoxide detection

Mitochondrial superoxide generation was investigated based on the oxidation and fluorogenic nucleic acid binding of a mitochondrial- and superoxide-specific probe (MitoSOX™, Invitrogen). Cells were stained according to the manufacturer's protocol and fixed with 4 % paraformaldehyde afterwards. Fluorescence was quantified using an Olympus BX51 microscope. Micrographs were quantified using ImageJ (NIH).

Scavenging of mitochondrial superoxide

HAEC were handled and transfected as described above. Upon transfection with siRNA the medium was supplemented with 1 µM mitoTEMPO (Sigma). Medium was replaced with fresh EGM-2 medium containing 1 µM mitoTEMPO once, before harvesting the lysates for expression analyses or staining the cells for fluorescence imaging.

Superoxide dismutase 2 (SOD2) activity

Mitochondrial fractions of HAEC were separated from whole cell lysates by centrifugation. Enzymatic activity of SOD2 in HAEC was assessed based on its capacity to dismutate superoxide radicals generated by xanthine oxidase under controlled conditions, using the Superoxide Dismutase Assay Kit (Cayman Chemical). Superoxide radicals were detected by colorimetric oxidation of tetrazolium salt to formazan dye. Any remaining activity of SOD1 and 3 was inhibited using potassium cyanide

(1 mM) according the manufacturer's instructions. Enzymatic activity was normalized to SOD2 protein expression.

Nitric oxide production

HAEC were seeded into a 96 well plate and transfected as described above. At time of the assay, cells were incubated with Krebs buffer containing 0.25 µM 4,5-diaminofluorescein diacetate (DAF-2, Sigma) in presence or absence of 10 µM of the unspecific nitric oxide synthase (NOS) inhibitor L-N^5-(1-Iminoethyl)ornithine hydrochloride (L-NIO, Sigma), or 0.25 µM of the DAF-2 negative control 4-aminofluorescein diacetate (4-AF-DA, Merck Millipore), respectively, for 20 min at 37 °C. Then L-arginine with or without calcium-ionophore (positive control) was added to the wells and fluorescence read at 490/525 nm (excitation/emission) to set the baseline. After 30 min the fluorescence was measured again and the percentage of nitric oxide increase calculated.

Statistics

Metric variables were assessed for distribution using Kolmogorov–Smirnov tests. For $n < 4$ non-parametric distribution was assumed. Different groups were compared using unpaired Student's t, Mann–Whitney, one-way ANOVA tests with Bonferroni multiple comparison post hoc tests or Kruskal–Wallis tests with Dunn's post hoc analyses, where applicable. p values are two-sided. Significance was accepted for an alpha-error <0.05. Data are presented as mean ± SEM, if not indicated otherwise. Statistical analyses were performed using GraphPad Prism 5 for Mac OS X (GraphPad Software).

Results

Transient knockdown of Sirt3 increases endothelial superoxide levels

siRNA-mediated transient knockdown of Sirt3 in human aortic endothelial cells (HAEC) reached an efficiency of 81 % on RNA- and 87 % on protein level (Fig. 1a). Intracellular superoxide levels were increased two-fold upon knockdown of Sirt3, as quantified using electron spin resonance spectroscopy (Fig. 1b). With Sirt3 being a mitochondrial deacetylase we used a mitochondrial- and superoxide-specific probe (MitoSOX™) to identify the cellular compartment of increased superoxide. Fluorescence imaging revealed a two-fold increase in mitochondrial superoxide levels, identifying mitochondria as the source of increased oxidative stress during transient knockdown of Sirt3 (Fig. 1c).

Loss of Sirt3 is associated with a mild superoxide-dependent impairment of endothelial function

To assess the functional relevance of elevated endothelial superoxide levels in the absence of Sirt3, aortic rings of *Sirt3*$^{-/-}$ and *wild-type* mice were explanted and endothelium-dependent relaxation was quantified in organ chamber baths. Surprisingly, aortic relaxation of *Sirt3*$^{-/-}$ mice in response to acetylcholine (ACh) was unaltered compared with *wild-type* controls (Fig. 2a). However, upon 12 weeks of high-cholesterol diet, known to increase oxidative stress [33], aortic relaxation of both genotypes was less sensitive to ACh at low dosages and showed an overall mild impairment in aortae of *Sirt3*$^{-/-}$ mice compared to *wild-type* controls (Fig. 2b). Scavenging endogenous superoxide by an excess of exogenous pegylated superoxide dismutase (PEG-SOD) improved the sensitivity to ACh of either genotype and abolished the impairment of aortic relaxation of high-cholesterol diet-fed *Sirt3*$^{-/-}$ mice compared to *wild-type* controls (Fig. 2c). ACh-induced aortic relaxation in both genotypes could be prevented by preincubation with the endothelial nitric oxide synthase (eNOS) inhibitor L-nitroarginine methyl ester (L-NAME), indicating endothelial NO-dependency (Fig. 2d, S1C). Concomitantly, complete relaxation of aortae of both genotypes in response to the exogenous NO donor sodium nitroprusside (SNP) further underlined endothelium-derived NO-dependency (Fig S1A, B). Of note, there was no significant difference in body weight between *wild-type* and *Sirt3*$^{-/-}$ mice (Fig S2). These findings suggest a mild, superoxide-dependent decline in aortic relaxation in the absence of Sirt3 upon a high-cholesterol diet.

Endothelial SOD2-specific activity is diminished whereas SOD2 expression is increased following transient knockdown of Sirt3

To unravel the mechanism underlying increased endothelial mitochondrial superoxide levels upon Sirt3 deficiency, we addressed SOD2-specific activity. Following transient knockdown of Sirt3 in HAEC, superoxide scavenging capacity of SOD2 was reduced by threefold compared with controls (Fig. 3a). Unexpectedly, expression levels of SOD2 were increased by 4.4 fold on RNA- and by 2.8 fold on protein level, respectively (Fig. 3c), thus compensating for the Sirt3-dependent loss of SOD2 specific activity; overall endothelial SOD2 activity, without normalizing to protein levels, was unchanged upon transient knockdown of *Sirt3* (Fig. 3b). Decreased SOD2-specific activity was associated with a trend towards SOD2 hyperacetylation (Fig. 3d). SOD2 nitrosylation was unchanged upon knockdown of Sirt3 (Fig. 3e), suggesting a Sirt3-dependent, deacetylation-mediated activation of endothelial

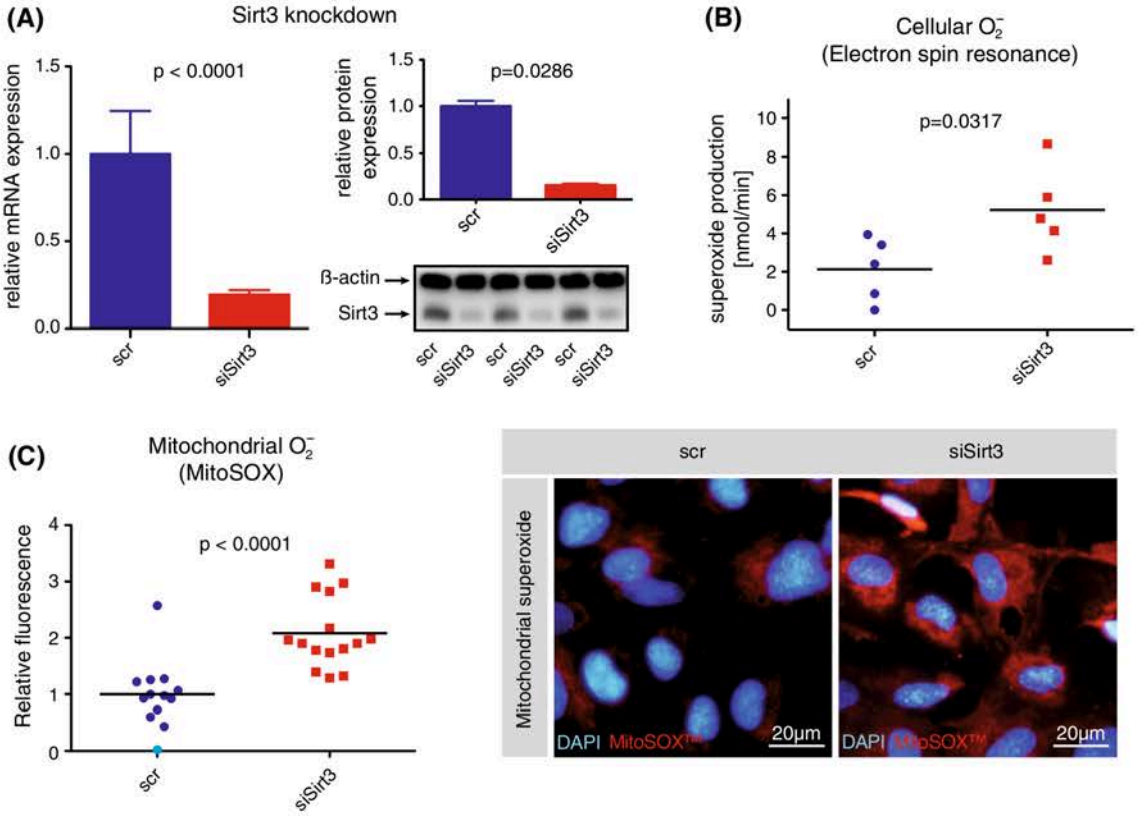


Fig. 1 Transient knockdown of Sirt3 increases endothelial superoxide levels. **a** Quantitative PCR of mRNA (*left*) and western blot analyses of protein (*right*) isolated from human aortic endothelial cells (HAEC) following siRNA-mediated knockdown of Sirt3. **b** Electron spin resonance (ESR) spectroscopy of live HAEC following siRNA-mediated knockdown of Sirt3 to quantify intracellular superoxide release. **c** Fluorescence imaging of HAEC following siRNA-mediated knockdown of Sirt3 and detection of mitochondrial superoxide (*red*) using MitoSOX™, a mitochondrial- and superoxide-specific probe; quantification on a per cell basis; representative micrographs show nuclei (*blue*) and mitochondrial superoxide (red, MitoSOX™); *scale bars* 20 µm. At least three independent experiments, each in biological triplicates, *scr* scrambled control, *DAPI* 4′-6-diamidin-2-phenylindol, **b** and **c** show medians and single data points

SOD2 under physiological conditions. Expression levels of other superoxide scavenging or decomposing enzymes, including SOD1, SOD3, catalase, thioredoxin 1, thioredoxin 2, thioredoxin-dependent peroxide reductase (PRDX3), and glutathione peroxidase were unaltered (Fig. 3f–h, Fig S3A–E). Accordingly, the expression level of endothelial superoxide-generating enzyme NADPH oxidase was unaffected by transient knockdown of Sirt3 (Fig S3F, G). Whereas no difference occurred in the cytosolic subunit $p47^{phox}$ (Fig S3F), we observed a slight increase in the mRNA level of the membrane-bound subunit $p22^{phox}$, which did not translate into an increased protein level (Fig S3G).

Nitric oxide generation is not affected by Sirt3 deficiency

Assessment of endothelial nitric oxide synthase (eNOS) uncovered unaltered overall expression levels (Fig S4A) and unchanged phosphorylation at Ser1177 (Fig S4B) upon Sirt3 knockdown. However, we observed a decreased phosphorylation of Thr495 following transient knockdown of Sirt3 (Fig S4C), equivalent to an increased enzymatic activity. Together with an increased coupling of eNOS monomers (Fig S4D) this may be a compensatory effect secondary to an increased mitochondrial ROS accumulation upon Sirt3 deficiency. To assess the functional relevance of increased eNOS activity, nitric oxide (NO) generation was assessed in presence or absence of L-NIO, an unselective nitric oxide synthase inhibitor, using DAF-2 diacetate. L-NIO successfully reduced NO generation, however, no significant difference was observed upon transient knockdown of Sirt3 compared with controls, neither in presence nor in absence of L-NIO (Fig S4E). Thus, increased eNOS activity upon Sirt3 deficiency does not generate a detectable rise in NO. Nonetheless, increased eNOS coupling may contribute to counteract increased ROS levels upon Sirt3 deficiency.

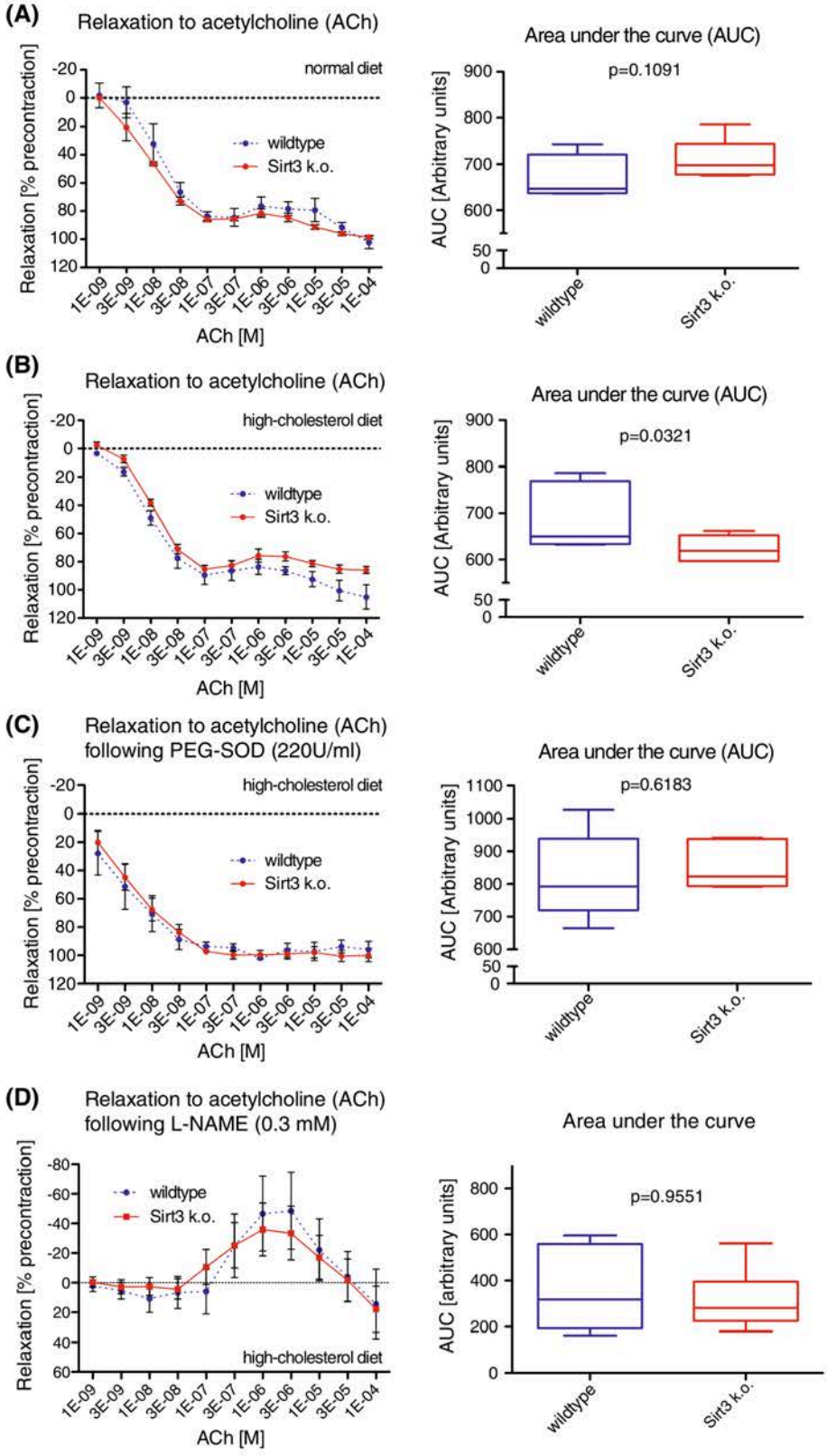


Fig. 2 Endothelial function is mildly impaired in Sirt3$^{-/-}$ mice in a superoxide-dependent manner. **a** Relaxation of aortic rings in response to increasing doses of acetylcholine (ACh) in *Sirt3*$^{-/-}$ compared with *wild-type* mice fed a regular chow. **b** As in **a** following 12 weeks of a high-cholesterol diet. **c** As in **b**, in presence of an excess of pegylated superoxide dismutase (PEG-SOD, 220 U/ml), scavenging superoxide. **d** As in **b**, following preincubation with L-NAME (0.3 mM, 30 min). $n = 5$–8 mice per group, 4–7 rings per mouse, quantification of the areas under the curve (AUC), *boxplots* show interquartile ranges, *whiskers* indicate minima and maxima

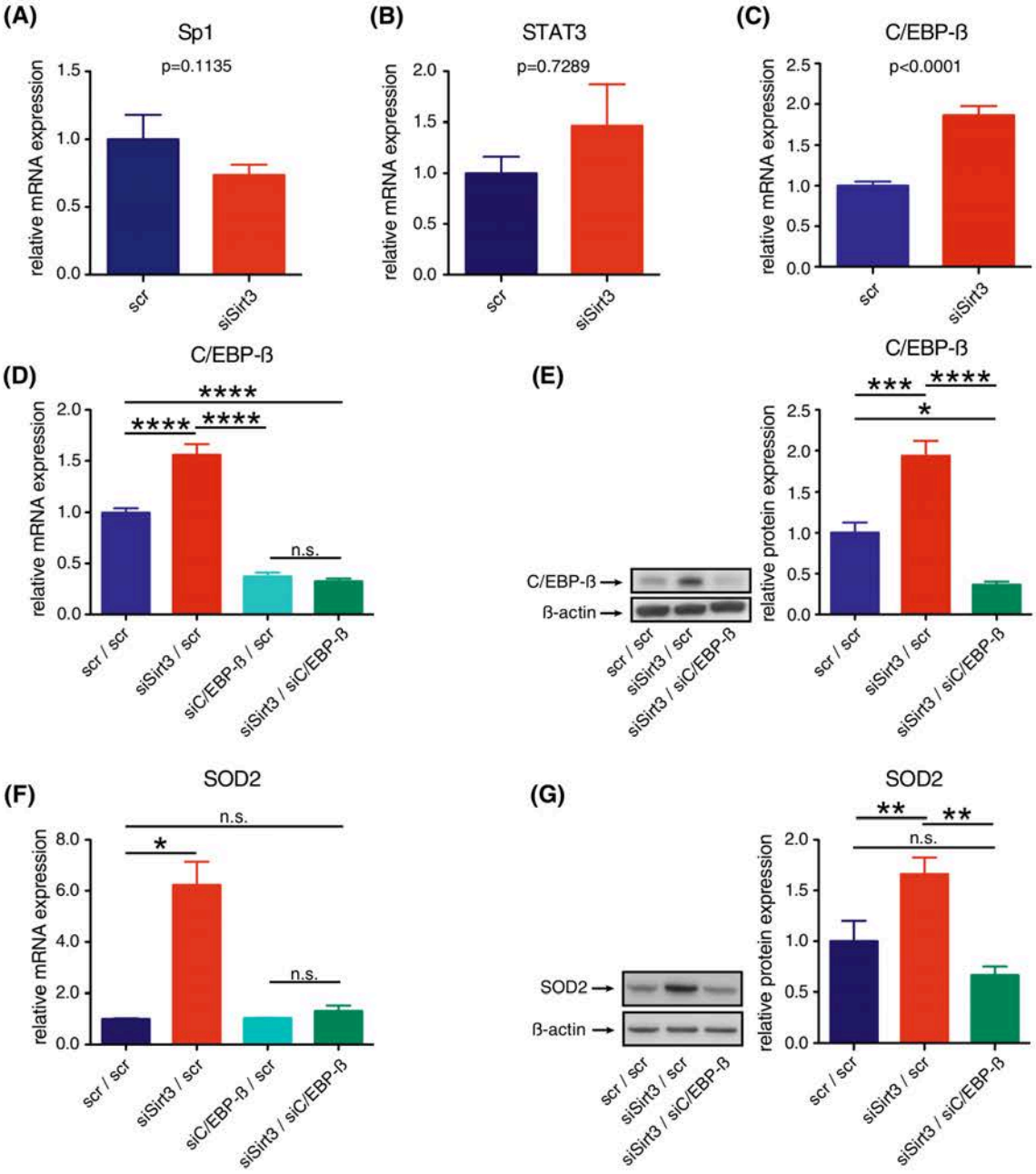


Fig. 4 Transcriptional induction of SOD2 upon transient knockdown of Sirt3 is C/EBP-β-dependent. **a–d** Expression analyses of the transcription factors **a** Sp1, **b** STAT3, **c**, **d** C/EBP-β using quantitative PCR in HAEC following siRNA-mediated knockdown of Sirt3, and **d** single or simultaneous transient knockdown of C/EBP-β and Sirt3, respectively. **e** Western blot analysis of C/EBP-β in HAEC following single and simultaneous transient knockdown of C/EBP-β and Sirt3, respectively. **f**, **g** Expression analyses of SOD2 in HAEC following single or simultaneous transient knockdown of C/EBP-β and Sirt3, respectively, using **f** quantitative PCR and **g** western blot analysis. At least three independent experiments in biological triplicates were performed, *scr* scrambled control, **d–g** *p* values indicate overall significance by Kruskal–Wallis (**d–f**) or one-way ANOVA tests (**g**), differences between single groups by Dunn's (**d–f**) or Bonferroni (**g**) post hoc tests are indicated separately: *n.s.* non-significant, $*p < 0.05$, $**p < 0.01$

transient knockdown of Sirt3. Interestingly, simultaneous knockdown of Sirt3 and C/EBP-β abrogated the transcriptional upregulation of SOD2, whereas single knockdown of C/EBP-β had no effect on SOD2 expression levels compared with controls (Fig. 4d–g).

Transcriptional induction of C/EBP-β is SOD2-dependent

To assess the role of SOD2 on the transcriptional induction of C/EBP-β we used a loss-of-function approach in HAEC.

Transient knockdown of SOD2 was associated with a significant increase in C/EBP-β transcription on RNA-level (Fig S5A), which translated into a trend towards increased protein levels of C/EBP-β (Fig S5B), indicating the existence of a direct feedback loop between SOD2 and its transcription factor C/EBP-β in endothelial cells. Sirt3 expression was unaltered (Fig S5 F, G).

Scavenging mitochondrial superoxide does not affect Sirt3-dependent transcriptional induction of SOD2

To investigate whether SOD2 induction upon Sirt3 deficiency is superoxide-dependent, mitochondrial superoxide was scavenged using the mitochondrial-targeted superoxide scavenger mitoTEMPO. Mitochondrial superoxide accumulation following knockdown of Sirt3 in HAEC was successfully blunted by mitoTEMPO, as assessed by fluorescence imaging after MitoSOX staining (Fig. 5a–c). Interestingly, SOD2 induction upon Sirt3 knockdown was unaffected by blunting mitochondrial superoxide accumulation (Fig. 5d). Transcriptional upregulation of C/EBP-β following knockdown of Sirt3 was also unaltered upon scavenging of mitochondrial superoxide (Fig. 5e). Translation to increased protein levels could not be observed, independent of mitochondrial superoxide (Fig. 5f).

Interruption of the physiological C/EBP-β-dependent feedback regulation of endothelial SOD2 exacerbates mitochondrial superoxide levels and culminates in endothelial cell death

To reveal the functional relevance of the C/EBP-β-dependent transcriptional feedback regulation of endothelial SOD2 upon Sirt3 deficiency, we assessed mitochondrial superoxide levels following single or simultaneous knockdown of C/EBP-β and Sirt3, respectively, compared with sham-transfected controls. Concomitant with the abrogation of the transcriptional induction of SOD2 following simultaneous knockdown of C/EBP-β and Sirt3, mitochondrial superoxide levels were further enhanced compared with single-knockdown controls (Fig. 6a, b). Transient knockdown of C/EBP-β alone had no effect on mitochondrial superoxide levels (Fig. 6a, b). Interestingly, we observed an increased cell death upon prolonged cultivation (40 h) following simultaneous knockdown of C/EBP-β and Sirt3 that occurred in none of the control conditions (Fig. 6c, d): Incubation for up to 40 h following knockdown led to a demise of the majority of cells (Fig. 6d), which we interpret as the consequence of increased oxidative stress.

Discussion

Principle findings

We identify Sirt3 as an important player in the homeostasis of endothelial mitochondrial superoxide. Under physiological conditions, endogenous endothelial Sirt3 appears to uphold SOD2 activity by maintaining its deacetylation for effective degradation of mitochondrial superoxide and thereby preserving normal endothelial function. Lack of Sirt3 is associated with a hyperacetylation of SOD2. A concomitant loss of SOD2 activity is, however, compensated for by a C/EBP-β dependent induction of SOD2 expression. The latter occurs independent of mitochondrial superoxide accumulation, yet potentially upon an initial transcriptional repression of SOD2 upon Sirt3 deficiency. Consequently, endothelial function is maintained under normal circumstances. Only upon a high-cholesterol diet, leading to increased oxidative stress, a mild, superoxide-dependent impairment of endothelial function can be unmasked. In vitro experiments in human aortic endothelial cells indicate that this may be due to an accumulation of mitochondrial superoxide secondary to a residual moderate imbalance of mitochondrial superoxide generation and detoxification. Since none of the superoxide scavenging or generating enzymes assessed were altered upon Sirt3 deficiency, we extrapolate that impaired mitochondrial function upon Sirt3 deficiency [38, 54] may increase mitochondrial superoxide formation.

Added value

Anti-oxidative effects of Sirt3 have been described in a variety of contexts including age-related hearing loss [49], embryogenesis [22], neuronal injury [10], exercise training [27], and cardiac hypertrophy [51]. In the majority of these settings, the protective effects of Sirt3 are mediated by an augmented radical scavenging through SOD2 and/or catalase. It remains controversial whether these effects are brought about by a transcriptional induction of either of these two ROS detoxification systems or by their Sirt3-dependent deacetylation and consecutive activation [8, 42, 51].

In the present study, we report on the role of endogenous Sirt3 in human aortic *endothelial* cells and its functional effects on *endothelium-dependent vasodilation* in a mouse model applying a genetic loss-of-function approach. Corroborating previous reports on anti-oxidative effects of Sirt3 [42], transient knockdown of *endothelial* Sirt3 abrogated the superoxide scavenging capacity of SOD2 and increased its acetylation.

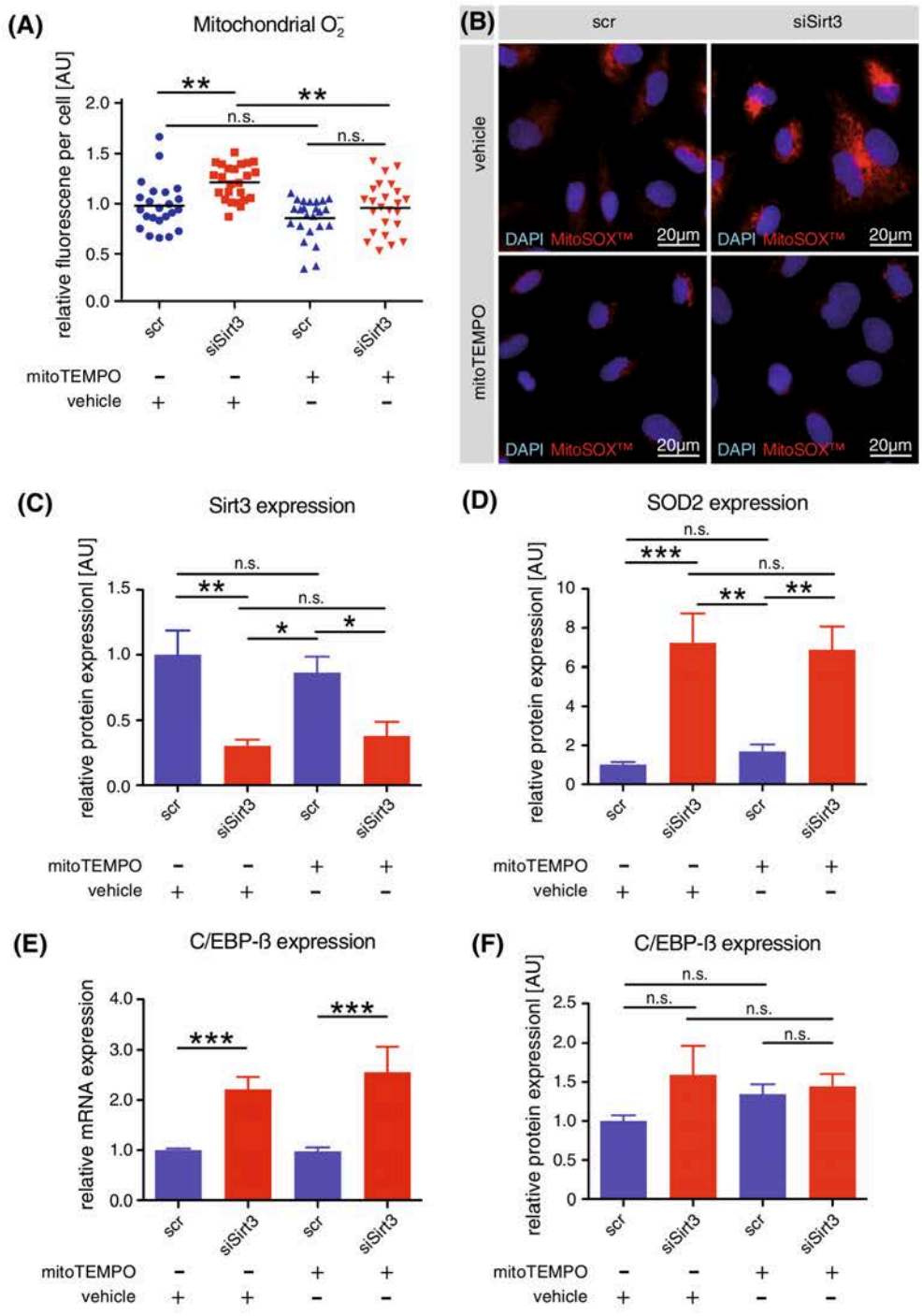


Fig. 5 Scavenging mitochondrial superoxide does not affect Sirt3-dependent transcriptional induction of SOD2. **a** Quantification of mitochondrial superoxide per cell, as visualized by MitoSOX™ staining, using fluorescence imaging of HAEC following transient knockdown of Sirt3 or transfection with scrambled siRNA (scr) in presence or absence of the mitochondrial-targeted anti-oxidant mitoTEMPO. **b** Representative micrographs of the setup described in **a**, showing nuclei (*blue*, DAPI) and mitochondrial superoxide (*red*, MitoSOX™). **c**, **d**, **f** Quantification of western blot analyses of Sirt3 (**c**), SOD2 (**d**), and C/EBP-β (**f**). **e** quantitative PCR of C/EBP-β. At least three independent experiments in biological triplicates were performed. *Scale bars* 20 µm, *DAPI* 4′-6-diamidin-2-phenylindol, *n.s.* non-significant, $^{**}p < 0.01$, $^{***}p < 0.001$

In contrast to previous data [51], we observed that transcription of endothelial SOD2 was not reduced but enhanced following transient knockdown of Sirt3, compensating for its decreased enzymatic activity. However, an initial transcriptional repression of SOD2 upon Sirt3 deficiency, preceding the C/EBP-β dependent

transcriptional induction of SOD2 cannot be excluded. Even though the drop in endothelial SOD2 activity upon Sirt3 deficiency slightly exceeded its transcriptional increase, we extrapolate that an excess in mitochondrial superoxide generation secondary to a Sirt3-dependent impairment of mitochondrial function [38] may contribute to the disequilibrium between mitochondrial superoxide generation and detoxification. Of note, expression levels of other endothelial ROS and reactive nitrogen species detoxification systems, including catalase and the thioredoxin system, were unaffected by transient knockdown of Sirt3. Therefore, a thioredoxin-mediated transcriptional induction of SOD2, as reported in yeast and primary human lung microvascular endothelial cells [11, 39] appears unlikely. Assessment of endothelial nitric oxide synthase (eNOS) uncovered a decreased phosphorylation of Thr495 as well as an increased coupling following transient knockdown of Sirt3, equivalent to an increased enzymatic activity [43, 52]. Importantly, NO generation remained unchanged, indicating that increased activity remains functionally irrelevant with regard to NO homeostasis. However, increased eNOS coupling may contribute to counteract increased ROS levels upon Sirt3 deficiency.

Moreover, our in vitro experiments identify mitochondria as the compartment exhibiting increased ROS in the absence of endothelial Sirt3, a finding, for which to date only indirect evidence exists [22, 54].

Elegant promoter studies identified that C/EBP-β is necessary to align with an intronic enhancer of SOD2 to facilitate its transcription in response to increased levels of intracellular ROS [6, 21, 30]. The differential regulation of C/EBP-β in response to transient Sirt3 knockdown and its acetylation-dependent binding capacity [6] prompted us to investigate the role of C/EBP-β in the transcriptional regulation of SOD2 in the absence of endothelial Sirt3. Abrogation of SOD2 induction upon simultaneous knockdown of both Sirt3 and C/EBP-β exacerbated mitochondrial superoxide accumulation and culminated in endothelial cell death after prolonged cultivation. Interestingly, we observed a bidirectional feedback regulation between C/EBP-β and SOD2 with an SOD2-dependent transcriptional induction of C/EBP-β and vice versa. This might indicate that an initial transcriptional repression of SOD2 upon Sirt3-deficiency, as has been reported by others [20, 24, 45, 51], in addition to a blunted SOD2 activity, may have preceded C/EBP-β dependent transcriptional induction of SOD2. Importantly, transcriptional induction of C/EBP-β was independent of mitochondrial superoxide. These findings highlight the functional relevance of this novel C/EBP-β-dependent transcriptional induction of SOD2 in absence of Sirt3 in human aortic endothelial cells.

The ex vivo assessment of endothelium-dependent vessel relaxation showed a rather atypically shaped relaxation curve; the initial sigmoidal shape is interrupted by a weak contraction, starting at acetylcholine levels around 1 μM, before reaching complete relaxation at 100 μM. Interestingly, this intermittent contraction was exacerbated in mice fed a high-cholesterol diet. This and the fact that this phenomenon disappeared in presence of PEG-SOD indicate that this intermittent contraction in response to higher acetylcholine dosages is an oxidative stress-dependent phenomenon, as we have observed previously [60].

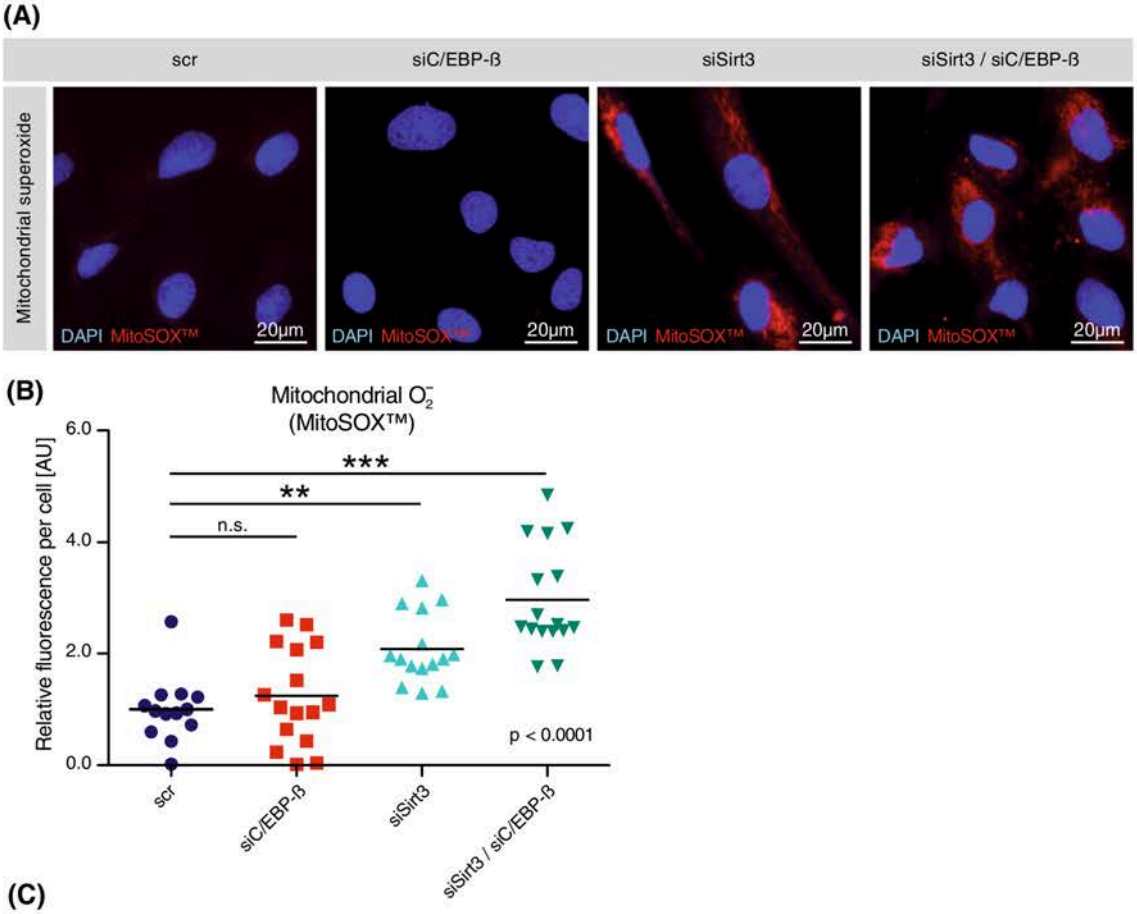
Potential limitations

This study has to be interpreted in light of the following limitations: The difference in endothelium-dependent vasorelaxation between the two genotypes is mild. However, rescue of this phenotype by scavenging of superoxide via exogenous pegylated superoxide dismutase proves superoxide-dependency. Furthermore, improvement of the sensitivity to ACh of both genotypes upon enhanced superoxide scavenging highlights the tight regulation of endothelial function by superoxide and NO and stresses the physiological relevance of Sirt3 and C/EBP-β in regulation of endothelial SOD2 activity and thus of endothelial function.

In light of the C/EBP-β dependent transcriptional compensation of the Sirt3-dependent loss of SOD2 activity in concert with unchanged expression levels of other superoxide generators or scavengers, our data do not provide an explanation for the increased mitochondrial superoxide levels upon Sirt3 deficiency. The extrapolation that a known Sirt3-dependent impairment of the mitochondrial function may enhance mitochondrial superoxide generation remains speculative and warrants further investigation.

In addition, we would like to point out that endothelial dysfunction in $Sirt3^{-/-}$ mice was observed only upon exposure to a high-cholesterol diet, known to induce oxidative stress [37]. In vitro, mitochondrial superoxide accumulation and differential SOD2 regulation upon Sirt3 deficiency was apparent under basal conditions. Though we assume that this is due to the nature of in vitro setups in general, extrapolation to our ex vivo data may be limited.

Moreover, endothelial-dependent vasodilation was assessed using mouse aortic rings in organ chamber baths, which is an ex vivo approach. Again, extrapolation to in vivo vascular function as well as to other species has to be done with caution.


(A)
scr
siC/EBP-ß
siSirt3
siSirt3 / siC/EBP-ß
Mitochondrial superoxide
DAPI MitoSOX™
20µm
(B)
Mitochondrial O_2^-
(MitoSOX™)
Relative fluorescence per cell [AU]
6.0
4.0
2.0
0.0
***
**
n.s.
p < 0.0001
scr
siC/EBP-ß
siSirt3
siSirt3 / siC/EBP-ß
(C)
scr
siSirt3
siC/EBP-ß
siSirt3 / siC/EBP-ß
brightfield, 17h post transf.
200µm
(D)
scr
siSirt3
siC/EBP-ß
siSirt3 / siC/EBP-ß
brightfield, 40h post transf.
200µm

◀**Fig. 6** Interruption of the physiological C/EBP-β-dependent transcriptional feedback regulation of SOD2 during transient knockdown of Sirt3 exacerbates mitochondrial superoxide formation and culminates in endothelial cell death. **a** Fluorescence imaging of HAEC following single and simultaneous transient knockdown of C/EBP-β and Sirt3, respectively. Representative micrographs show nuclei (*blue*) and mitochondrial superoxide (red, MitoSOX^{TM}), the latter visualized by the mitochondrial- and superoxide-specific fluorescent MitoSOX^{TM} probe. *Scale bars* 20 μm. **b** Quantification of mitochondrial superoxide per cell; medians and single data points are shown. **c**, **d** Representative brightfield phase-contrast micrographs of cultured HAEC 17 h (**c**) and 40 h (**d**) after transient knockdown of Sirt3 and C/EBP-β, either alone or in combination; *scale bars* 200 μm. At least three independent experiments in biological triplicates were performed, *scr* scrambled control, *DAPI* 4′-6-diamidin-2-phenylindol, *n.s.* non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Conclusions and implications

The current data indicate a protective role of endogenous endothelial Sirt3 in mice fed a high-cholesterol diet, maintaining endothelium-dependent vasorelaxation. The in vitro findings suggest that a novel C/EBP-β-dependent rescue mechanism diminishes *Sirt3*-dependent endothelial dysfunction under physiological conditions (normal diet).

We have reported Sirt3-mediated protection from accelerated weight gain and a decline in metabolic flexibility [55], two important risk factors of human cardiovascular diseases [5]. Our current findings identify an interplay of Sirt3, SOD2, and C/EBP-β in the endothelial redox system. Endothelial dysfunction is independently associated with future adverse cardiovascular events [16, 17, 36, 40, 48]. Further research is warranted to better understand the putatively protective role of Sirt3 in human cardiovascular disease.

Acknowledgments This work was funded by the Swiss National Science Foundation to JA (31003A-140780), to TFL (310030-135815), and to CMM (146923, 140336), the National Institute of Health (US) to JA (R01AG043930), Systems X (SysX.ch) to JA (2013-15), the University Research Priority Program Integrative Human Physiology at the University of Zurich to TFL and CMM, Matching Funds by the University of Zurich to SW and CMM, the Ecole Polytechnique Fédérale de Lausanne to JA, and the Zurich Heart House—Foundation for Cardiovascular Research, Zurich, Switzerland.

Compliance with ethical standards

Conflict of interest None.

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Mild endothelial dysfunction in Sirt3 knockout mice fed a high-cholesterol diet

Protective role of a novel C/EBP-ß-dependent feedback regulation of SOD2

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Supplementary Material / Online Resource

Running title: *Sirt3 in Endothelial Function*

Key words: *Sirt3, oxidative stress, SOD2, C/EBP-ß, endothelial function*

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Figure S1 – *Aortic relaxation is endothelium- and nitric oxide (NO)-dependent*

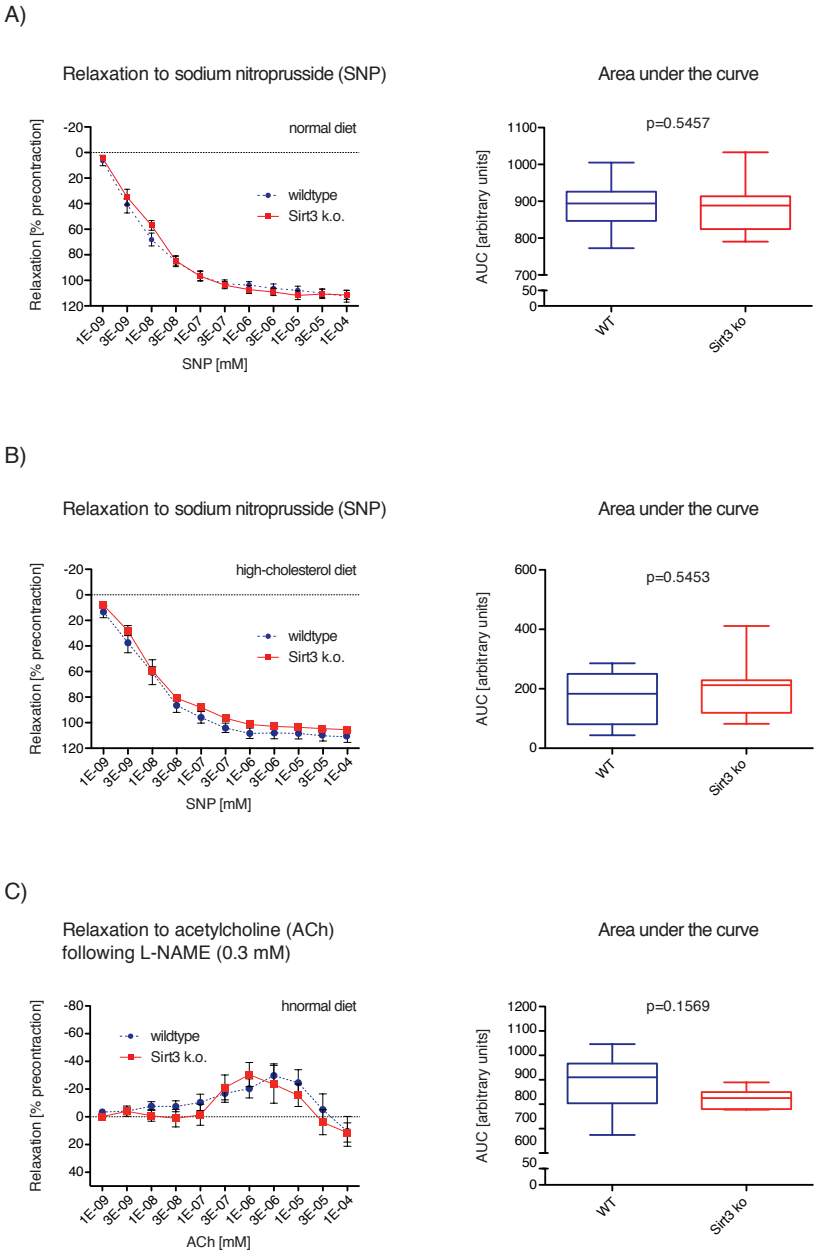

Figure S1 – Relaxation of aortic rings from *wild-type* and *Sirt3*$^{-/-}$ mice in response to sodium nitroprusside (SNP) following a normal diet (A) or a high cholesterol diet (B). (C) Relaxation of aortic rings from *wild-type* and *Sirt3*$^{-/-}$ mice fed a normal diet in response to acetylcholine (ACh) after preincubation with L-nitroarginine methyl ester (L-NAME), an inhibitor of endothelial nitric oxide synthase. n = 9 to 11 per group, quantification of the areas under the curve (AUC), boxplots show interquartile ranges, whiskers indicate minima and maxima.

Figure S2 – *Body weight does not differ between Sirt3*$^{-/-}$ and *wildtype* controls

A)

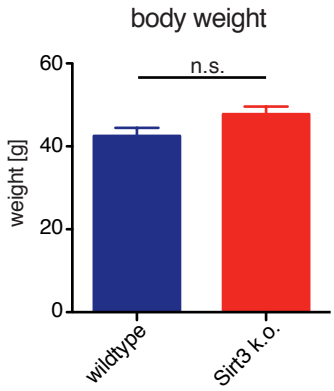

Figure S2 – (A) Body weights of *wild-type* and *Sirt3*$^{-/-}$ mice before subjecting them to organ chamber experiments.

Figure S3 – *Expression of glutathione peroxidase, xanthine oxidase, thioredoxin 1 and 2, and thioredoxin-dependent peroxide reductase are unaltered following transient knockdown of Sirt3*

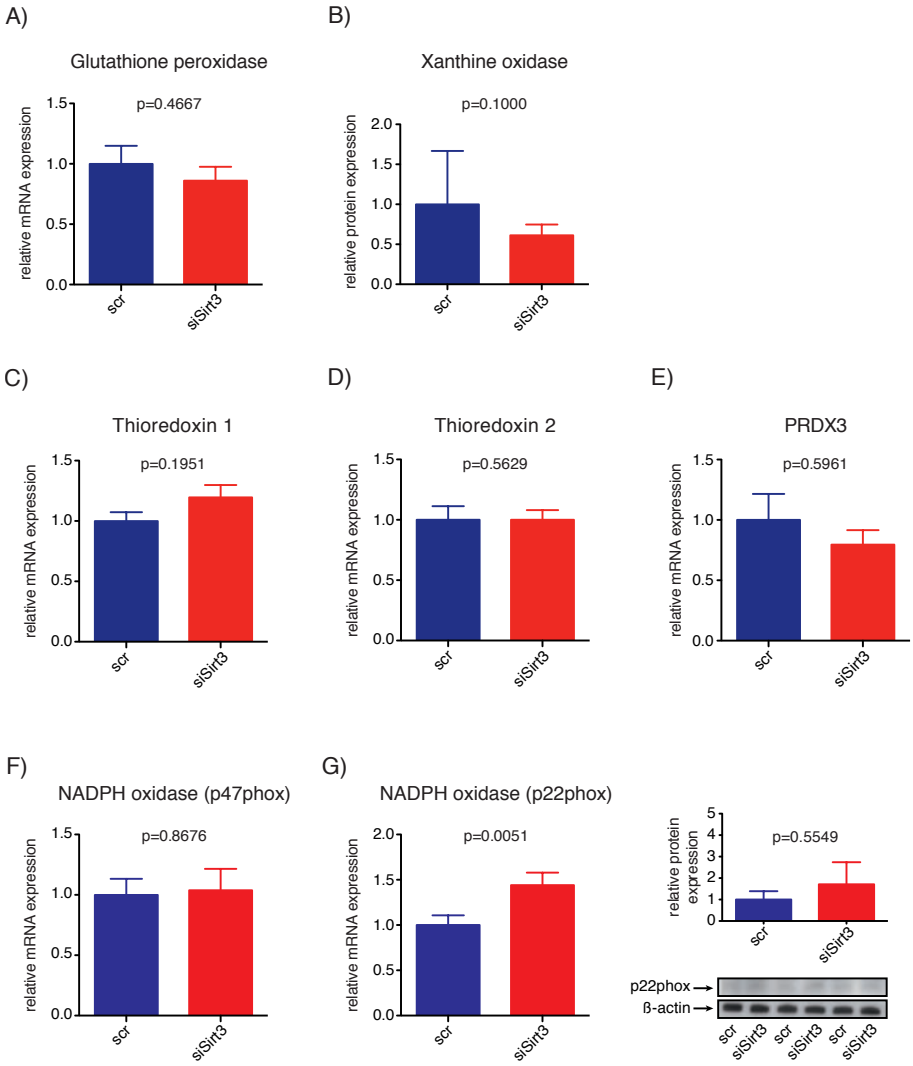

Figure S3 – Expression analyses of (A) glutathione peroxidase, (B) xanthine oxidase, (C) thioredoxin 1, (D) thioredoxin 2, (E) thioredoxin-dependent peroxide reductase (PRDX3), and (F, G) NADPH oxidase subunits $p47^{phox}$ and $p22^{phox}$ in HAEC following transient knockdown of Sirt3, using quantitative PCR (A, C-G left panel) and western blot analysis (B, G right panel), respectively. At least three independent experiments in biological triplicates were performed. Scr = scrambled control.

Figure S4 – *Nitric oxide (NO) generation is not affected by Sirt3 deficiency*

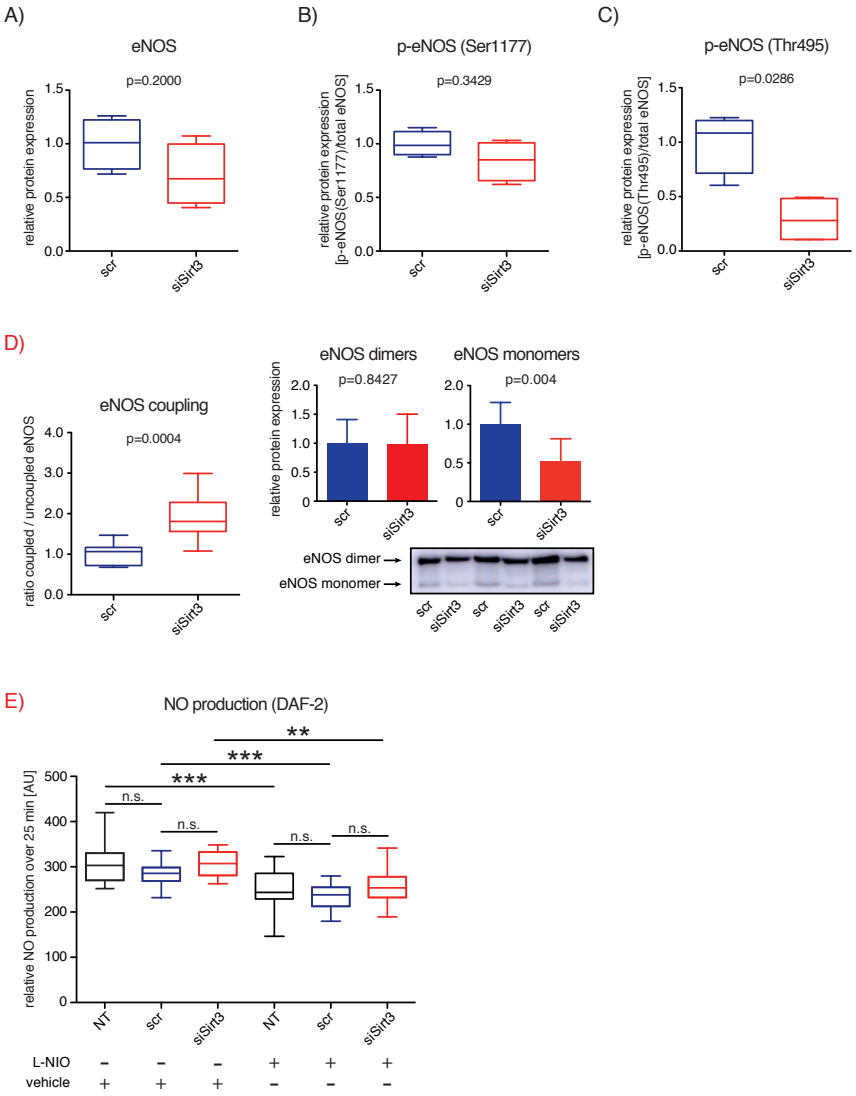

Figure S4 – Expression analyses (western blot) of (A) total eNOS, (B) eNOS phosphorylated at serine 1177 (p-eNOS(Ser1177)), and (C) eNOS phosphorylated at threonine 495 (p-eNOS(Thr495)) in HAEC upon knockdown of Sirt3 or control transfection using scrambled siRNA (scr). (D) Western blot analyses of eNOS un-/coupling upon knockdown of Sirt3 or control transfection using scrambled siRNA (scr). (E) Nitric oxide production using DAF-2 diacetate in HAEC upon knockdown of Sirt3, control transfection using scrambled siRNA (scr) or non-transfected (NT) controls. Each condition was assessed with or without (vehicle) L-NIO, a non-selective nitric oxide synthase inhibitor. ***) $p<0.001$, **) $p<0.01$, n.s. = not significant. Boxpots show interquartile ranges, whiskers indicate minima and maxima.

Figure S5 – *Loss of SOD2 induces transcription of C/EBP-ß*

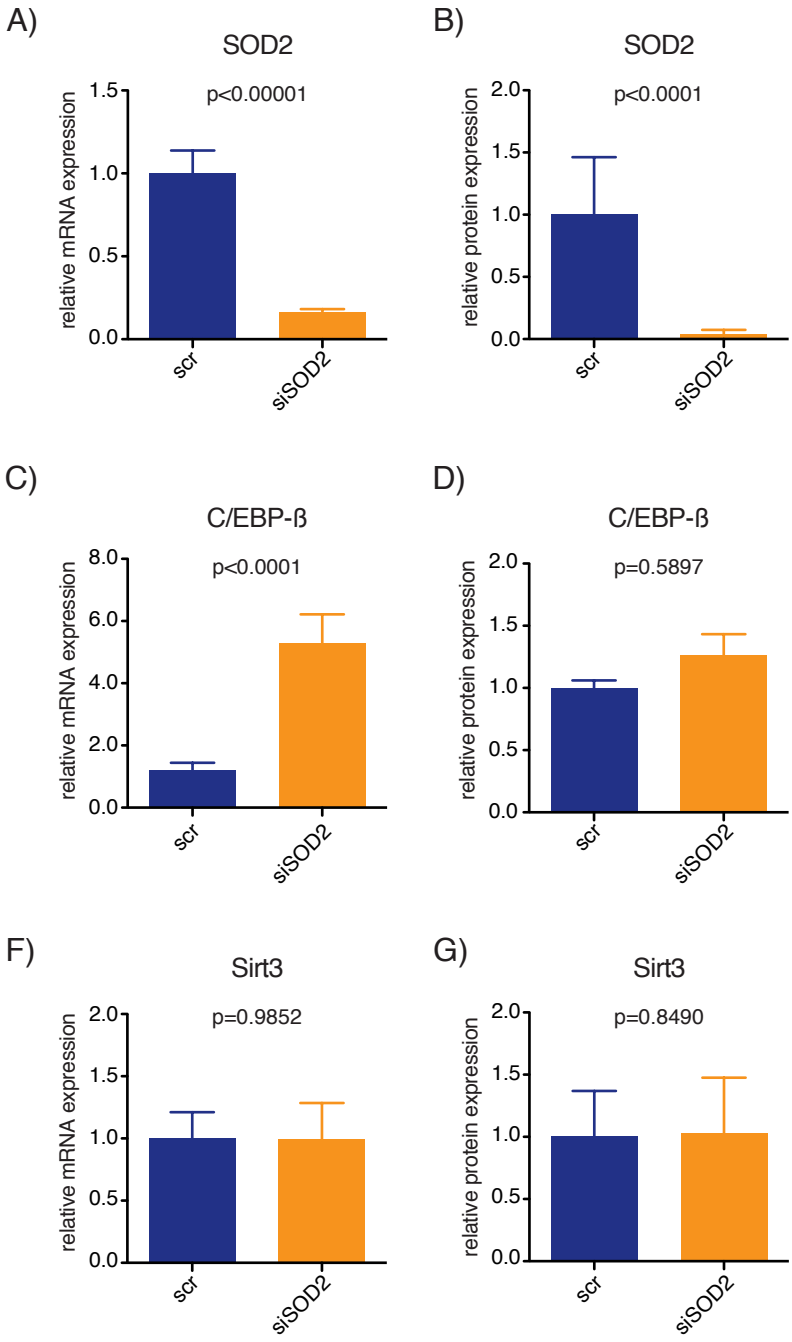

Figure S5 – Expression analyses using quantitative PCR (left hand side) and western blot analysis (right hand side) of SOD2 (A, B), C/EBP-ß (C, D), and Sirt3 (F, G) of HAEC following transient knockdown of SOD2 (siSOD2) or control transfection with scrambled siRNA (scr). At least three independent experiments in biological triplicates were performed.

6.3 Loss of Sirt3 accelerates arterial thrombosis by increasing formation of neutrophil extracellular traps and plasma tissue factor activity

The manuscript is blocked from publication in the Zentralbibliothek Zürich for 1 year.

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Daniel S. Gaul, Julien Weber, Lambertus J. van Tits, Susanna Sluka, Lisa Pasterk, Martin F. Reiner, Natacha Calatayud, Christine Lohmann, Roland Klingenberg, Felix C. Tanner, Giovanni G. Camici, Johan Auwerx, François Mach, Stephan Windecker, Nicolas Rodondi, Thomas F. Lüscher, Stephan Winnik*, and Christian M. Matter*

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Status of the manuscript:
Submitted to: European Heart Journal (EHJ)
Submission date: 14 June 2017

The attached manuscript is the version that was submitted to EHJ.

Author contributions Daniel S. Gaul:

- Planning, data acquisition, analysis, statistical evaluation, and interpretation of experiments
- Contributions to the individual figures:
    - Figure 1: contributed subfigure 1C
    - Figure 2: contributed subfigure 1A-C
    - Figures 3 and 4: contributed the whole figures
    - Figure 5: contributed subfigures 5A and 5C
    - Figure 6: contributed figure 6A and 6B
    - Figure 7: Contributed whole figure
    - Supplemental Figure 1 (S1): contributed the whole figure
    - Supplemental Figure 2 (S2): contributed subfigures S2A-C in collaboration with Martin F. Reiner
    - Supervision of visiting PhD student Lisa Pasterk, who contributed Figure 5B and 5D.
- Writing, editing, and proofreading of the manuscript

6.4 Endothelial Sirt6 deficiency accelerates arterial thrombosis by upregulating tissue factor and pro-inflammatory cytokines

The manuscript is blocked from publication in the Zentralbibliothek Zürich for 1 year.

Authors:
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Status of the manuscript:
Manuscript in preparation (experiments in progress)

Author contributions Daniel S. Gaul:
- Conception and design of the study
- Planning, organization, and, in collaboration with Julien Weber, generation of endothelial specific Sirt6 knockout mouse line
- Planning, data acquisition, analysis, statistical evaluation, and interpretation of experiments
- Contributions to the individual figures:
    - Figure 1: contributed the whole figure
    - Figure 2: contributed figure 2B-2E in collaboration with Nicole R. Bonetti
    - Supervision of Master student Natacha Calatayud, who contributed Figure 3 and 4 as a part of her Master project: 'Sirtuin-6 protects human aortic endothelial cells from a pro-thrombotic phenotype'
- Writing, editing, and proofreading of the manuscript

7 Discussion

7.1 Main findings

In the first part of this dissertation, we investigated the effects of a global Sirt3 deletion in a mouse model in atherosclerosis, endothelial function, and arterial thrombosis. We hypothesised that Sirt3 deletion accelerates atherosclerosis, induces endothelial dysfunction and enhances thrombosis due to an increase in oxidative stress.

In the second part, we focused on the role of Sirt6 in thrombosis. We hypothesised that Sirt6 depletion in the endothelium increases arterial thrombosis through the activation of NF-κB and AP-1 pro-inflammatory pathways in the endothelium.

Sirt3 in atherosclerosis

To assess the effects of Sirt3 in atherosclerosis, we generated $Sirt3^{-/-}$ mice on the background of an LDL-R knockout atherosclerotic mouse model and fed them a high-cholesterol diet for 12 weeks to induce atherosclerosis. Interestingly, although levels of the systemic oxidative stress marker malondialdehyde were increased in these mice (**Figure 11**), deletion of Sirt3 did not affect plaque burden, fibrous cap thickness, necrotic core diameter, or plaque macrophage and T-cell infiltration. However, loss of Sirt3 was coupled to an accelerated weight gain and impaired capability to cope with rapid changes in nutrient supply.

Sirt3 in endothelial function

Secondly, we fed $Sirt3^{-/-}$ mice a high-cholesterol diet for 12 weeks and subsequently evaluated endothelial function. We showed that Sirt3 deficiency blunts SOD2 activity and increases levels of superoxide in endothelial cells *in vitro.* However, endothelial function assessed by aortic relaxation capacity was only mildly impaired in $Sirt3^{-/-}$ mice (**Figure 11**). Supplementing the aortae with pegylated SOD, rescued the impaired relaxation capacity. As a potential cause for the mild effect, we identified a novel C/EBP-β-dependent feedback upregulation of SOD2, which is able to protect the endothelium from oxidative stress in absence of Sirt3. Inactivating this rescue mechanism enhanced endothelial cell death.

Sirt3 in arterial thrombosis

Furthermore, we tested the effect of Sirt3 deficiency on laser-induced arterial thrombosis in Sirt3$^{-/-}$ mice stimulated with LPS. Time to thrombotic occlusion in these mice was reduced by half compared to the control group. Moreover, clot formation was accelerated and clot stability increased. We discovered higher levels of circulating TF in the plasma and an increased NET formation rate in Sirt3-deficient neutrophils as reasons for accelerated thrombosis (**Figure 11**). In line with this observation, transcription of SOD2 in murine Sirt3$^{-/-}$ neutrophils was decreased and leukocytes of patients showed a reduction in SOD2, as well as Sirt3, after STEMI.

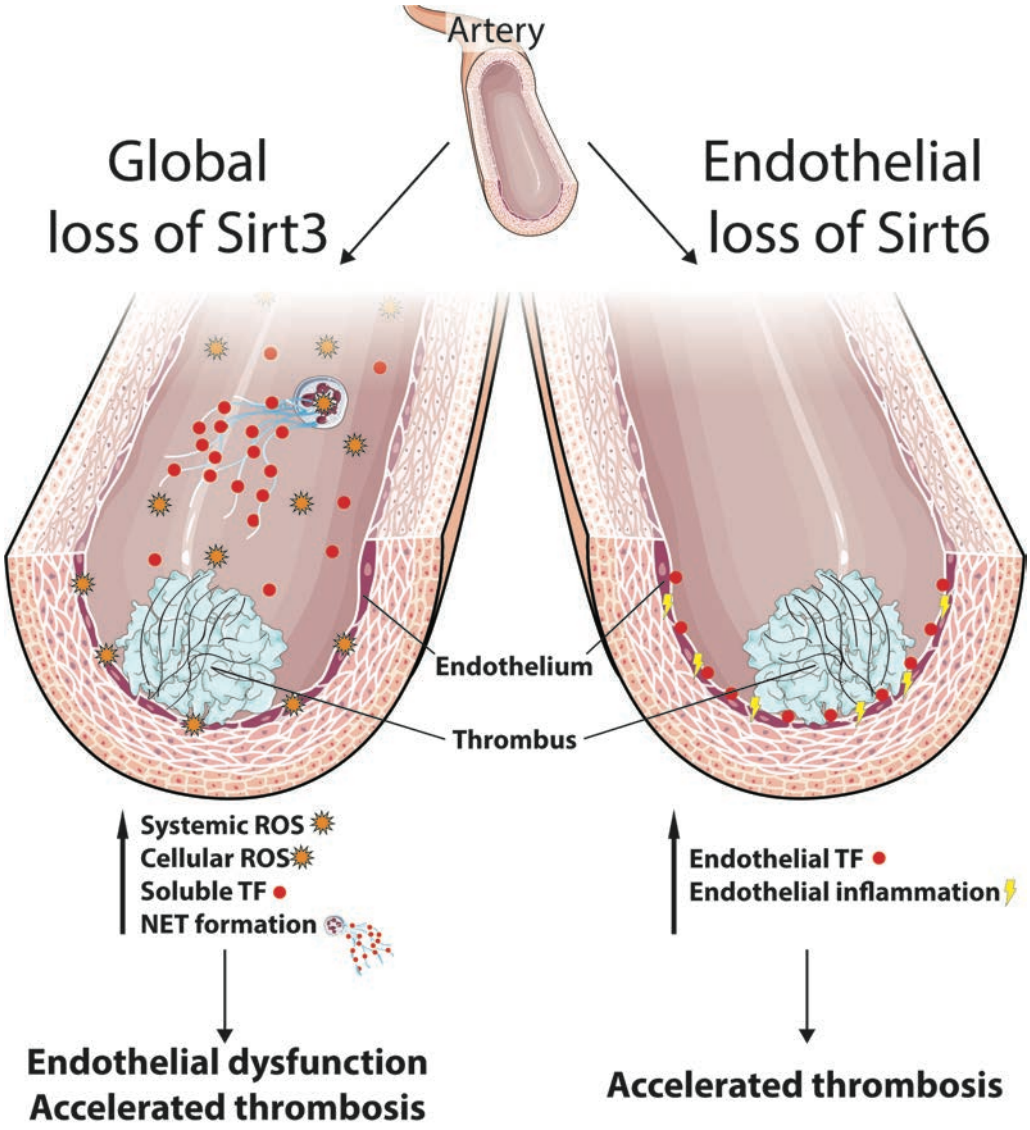

Figure 11. The effects of global loss of Sirt3 and endothelial loss of Sirt6 on the vasculature. Left: Global loss of Sirt3 leads to increased systemic reactive oxygen species (ROS), increased levels of circulating soluble tissue factor (TF) and increased formation of neutrophil extracellular traps (NETs). Furthermore, ROS in endothelial mitochondria is increased. All these changes lead to endothelial dysfunction and accelerated arterial thrombosis. Atherosclerosis was not affected by Sirt3 deficiency in our setting. **Right**: Endothelium-specific deletion of Sirt6 leads to accelerated thrombosis via increased TF expression and inflammatory signalling in endothelial cells.

Sirt6 in arterial thrombosis

Finally, we analysed the effect of endothelium-specific Sirt6 deficiency on arterial thrombosis. Time to thrombotic occlusion in endothelial Sirt6$^{-/-}$ mice occurred 45% faster compared to control mice, after inducing arterial thrombosis with a laser. *In vitro,* Sirt6-deficient HAECs exhibited increased TF transcription, protein expression and activity, along with transcriptional upregulation of pro-inflammatory cytokines that are induced by NF-κB and AP-1 transcription factors (**Figure 11**).

7.2 Key findings in comparison to current literature

Added value of our Sirt3 loss-of-function data

Opposing our hypothesis, Sirt3 deficiency did not affect atherosclerosis and induced only mild endothelial dysfunction, although we could prove that loss of Sirt3 is increasing oxidative stress systemically and in endothelial cells. We identified a novel C/EBP-β-dependent feedback mechanism that explains how the endothelium is protected from ROS-induced endothelial dysfunction. The same mechanism could explain a lack of difference between Sirt3$^{-/-}$ and control mice in atherosclerosis: when Sirt3 is lacking, the compensatory mechanism protects the endothelium from dysfunction, which prevents increased cellular adhesion of leukocytes to the endothelium and subsequently accelerated progression of atherosclerosis. Furthermore, our findings show that loss of Sirt3 on an atherogenic background impairs metabolic adaptation and causes weight gain. These findings are in line with previous ones that assigned Sirt3 a role in the development of metabolic syndrome.[164]

To date, we are the only group that investigated Sirt3 in atherosclerosis and arterial thrombosis, but other groups also examined its implication in endothelial function. A study, which was published only one month before ours, confirmed that loss of Sirt3 increases superoxide levels in endothelial cells.[201] However, as opposed to our study, Yang and associates showed a more pronounced impairment of endothelial relaxation capacity in Sirt3$^{-/-}$ mice. This is likely due to the different model they chose. First of all, they used the 129-mouse strain for their experiments, while we used C57BL/6 mice. The strain can immensely influence the outcome of an experiment. Indeed, a study from 1990 showed, that when mice of 16 different genetic backgrounds were subjected to an atherogenic diet, some strains, including C57BL/6, were very susceptible to atherosclerosis, while others were completely resistant.[202] More specifically, when comparing aortic cross sections of C57BL/6 and 129 mice after 14 weeks

of atherogenic diet, C57BL/6 mice exhibited a mean lesion area of 4200µm^2 per section, while 129 mice showed a mean area of only 350µm^2. Our strong interest towards the role of Sirt3 in atherosclerosis, together with clear proof from the literature, stating that C57BL/6 background is among the best models for this disease, served the basis for choosing these mice for our projects. Secondly, Yang and their team used a different stimulus to activate the endothelium. They used a model of obesity by feeding their mice a 45% high-fat diet for 24 weeks, in contrast to our 12-week 1.25% high-cholesterol diet. While no significant difference in body weight between Sirt3$^{-/-}$ and wildtype could be observed in our model, the weight of 129-Sirt3$^{-/-}$ mice was increased by 26.6% compared to respective controls.[201] As obesity presents a major risk factor for endothelial dysfunction, it makes sense that such a pronounced increase affects endothelial function.[203]

Our results assessing Sirt3 in arterial thrombosis support our hypothesis and add neutrophils as new protagonist cells in which Sirt3 plays a protective role. Since formation of NETs is dependent on generation of ROS, it is likely that Sirt3 is protecting neutrophils from NET release by mediating ROS via SOD2 and CAT, as previously described in other contexts.[161-163] Interestingly, a recent discovery associated loss of Sirt3 with an impaired recovery after myocardial ischaemia.[204] Taking into account the detrimental role of NETs after reperfusion, increased formation of NETs may explain why the recovery after ischaemia in Sirt3-deficient mice is impaired.[129]

Added value of our Sirt6 loss-of-function data

The second proposed hypothesis, stating that endothelium-specific loss of Sirt6 increases thrombosis via activation of NF-κB and AP-1 pathways, was verified by our results. However, since it is still work in progress, we cannot confidently conclude if or which of the two pro-inflammatory transcription factors plays a more pronounced role in thrombosis induced by endothelial Sirt6 deficiency. All the identified upregulated pro-inflammatory targets can be controlled by NF-κB and AP-1. To date, there are no publications dealing with Sirt6 in thrombosis. However, a role for Sirt6 in endothelial dysfunction and atherosclerosis has been described.[199,200,205,206] Since these studies specifically investigated endothelial cells, the results can be well compared to our findings. It has been demonstrated *in vitro* that human umbilical vein endothelial cells lacking Sirt6 show a pro-inflammatory phenotype with an increase in expression of cell adhesion

molecules.[200,205] These observations were likely triggered by an increase in NF-κB expression and signalling and are in line with our results.[200]

On the other hand, one of the studies also showed an increase in pro-inflammatory interleukins (IL) 1β, 6 and 8, which is something we could not observe.[200] In fact, our findings suggest, that transcription of IL-6 and IL-8 is downregulated in Sirt6-deficient cells. The main reason for the difference may be that in the published study, LPS is used to stimulate endothelial cells. The use of this bacterial endotoxin may trigger an increased release of interleukins that is even more pronounced in the absence of Sirt6. Furthermore, we used human aortic endothelial cells, whereas our colleagues used umbilical vein endothelial cells. It cannot be excluded, that Sirt6 plays different regulatory roles in the different cell types. Finally, *in vivo* studies using heterozygous deletion or gene knockdown of Sirt6 on an atherosclerotic background sustain our findings, that VCAM-1 and ICAM-1 are upregulated in the absence of Sirt6.[199,206]

7.3 Potential limitations

As already described, Sirt3 deficiency per se is not sufficient to induce a strong phenotype in mice, unless the system is challenged by another stimulus.[148,150] For our studies of atherosclerosis and endothelial function we chose a high-cholesterol diet as a stimulus because it induces atherosclerosis and activates the endothelium. This diet may have blunted the effect of Sirt3 in control mice, as the activity of all sirtuins is inhibited upon caloric excess. However, a complete inhibition of Sirt3 is highly unlikely, since our analyses showed less global mitochondrial acetylation in controls compared to Sirt3-depleted mice.

In the endothelial function study, we used a high-cholesterol diet in $Sirt3^{-/-}$ mice with intact LDL-R. Studies investigating hypercholesterolemia in mice showed that $LDL\text{-}R^{+/+}$ mice did not have increased cholesterol levels when fed a moderate cholesterol diet.[207] This indicates that $Sirt3^{-/-}$ mice may still cope rather well with the high-cholesterol diet and possibly suffer merely from moderate stress. Our findings show that ROS that is induced by this stress can be scavenged via a feedback-upregulation of SOD2 in endothelial cells. Taken together, these findings suggest that feeding a high-cholesterol diet may have been too weak of a stimulus to induce a Sirt3-mediated change in the endothelium.

A stronger stressor was used to assess the function of Sirt3 in arterial thrombosis. Sirt3$^{-/-}$ mice were stimulated with LPS, a bacterial endotoxin that activates leukocytes and triggers formation of ROS.[208] LPS may not be occurring in all cases of atherothrombosis and classically this disease is seen as a non-infectious inflammatory disease. Nevertheless, there are studies challenging this view by associating the gut microbiome with cardiovascular diseases and neutrophil ageing, indicating that endogenous bacterial endotoxins play a role in CVD.[209,210] Furthermore, neutrophil activation can also be triggered by the activated endothelium and platelets, indicating that a pathogenic stimulus is not necessary to induce neutrophil reactions in atherothrombosis.[111,112]

As Sirt6 deficiency has a much stronger effect in mice than deletion of Sirt3, no additional stimulus was used for these experiments. We generated an endothelium-specific knockout of Sirt6 to assess the function of this nuclear sirtuin in thrombosis. In earlier studies, Cre-recombinase expressed under the Tie2 promoter was used to achieve an endothelium-specific deletion. It was later shown, however, that Tie2 is also expressed in monocytes/macrophages, indicating that Tie2-mediated knockouts were not specific to the endothelium.[211] Consequently, we employed a model that deleted exons of Sirt6 by use of a Cre-recombinase, expressed under the vascular endothelial Cadherin (VE-Cadh) promoter, which is currently thought of as the best method to achieve an endothelial knockout.[212] Yet, it may be possible that other cell types also express VE-Cadh. Furthermore, in the current state of the project, it is not completely clear, if the generated Sirt6 knockout really worked as we anticipated. Additional studies to characterise and prove the endothelial deletion of Sirt6 are necessary.

A more general limitation of our study may be the age of the experimental mice. We used relatively young mice for our studies, whereas CVD in humans usually develops over long time periods and thus leads to complications only in aged individuals. Hence, the results may not be representative of the events in an aged organism. In fact, in an aged mouse, more pronounced effects of loss of Sirt3 or Sirt6 in atherothrombosis may be expected.

Finally, we only used male mice in our studies, which may limit the extent to which the results are relevant to females.

7.4 Implications and outlook

Sirt3 reduces chances of cardiovascular risk factor development

Even though Sirt3 does not appear to affect atherosclerotic plaques, we showed that Sirt3-deficient mice on an atherosclerotic background have difficulties in maintaining metabolic homeostasis and show accelerated gain of weight. As both metabolic disorders and obesity are major risk factors for CVD, Sirt3 may play an important role in the prevention of cardiovascular risk factor development.[143,203] This does not affect atherosclerosis immediately, but over time. Thus, for future studies of Sirt3 in atherosclerosis, it would be interesting to use aged Sirt3-deficient mice. To additionally reduce a possible blunting effect of a high energetic diet, such as high-cholesterol diet, on the sirtuins, the LDL-R$^{-/-}$/ApoB100 mouse model of atherosclerosis could be used. In this mouse model, the capability of mice to edit apolipoprotein B (apoB) mRNA is impaired, so they can only synthesise apoB100, which remarkably increases LDL levels and induces severe atherosclerosis in animals on a normal chow diet.[213] Finally, as our results revealed, Sirt3$^{-/-}$ mice have problems to adapt to fast changing nutrient supply. This finding could be used to induce additional stress by fasting the mice once or in cycles, before analyses.

Sirt3 protects the endothelium from mitochondrial ROS

Our results provide insights into a novel rescue mechanism that protects endothelial cells from ROS in absence of Sirt3. This highlights the importance of ROS scavenging in the vasculature. As mentioned above, we may have used a relatively weak stressor in our system. Using a stronger stimulus, as was done by another group, affected endothelial function in Sirt3$^{-/-}$ animals to a greater extent, compared to our model.[201] We can conclude that Sirt3 plays a protective role in the endothelium. For future research, it would be interesting to see, if Sirt3 overexpression preserves endothelial function in mice. Furthermore, diabetes is a major risk factor for endothelial dysfunction.[214] Since several studies linked Sirt3 to the regulation of insulin sensitivity and protection from insulin resistance, it would be particularly interesting to investigate endothelial function in Sirt3-deficient diabetic mice.[156,201,215]

Sirt3 regulates NET formation

As described, NETs are implicated in a wide range of diseases, including CVD. Investigating arterial thrombosis, our findings demonstrate that Sirt3 is able to prevent NET formation in neutrophils. Thus, activation of Sirt3 may reduce NET

formation in disease contexts and improve patient outcome. However, it remains elusive whether this would also affect neutrophil anti-infectious mechanisms. Yet, it could be studied using a neutrophil-specific Sirt3 overexpression approach. Sirt3 could be overexpressed under the promoters of neutrophil-specific CD18 integrin or myeloid-related protein 8, as used before for specific knockout approaches.[216,217]

Sirt6 protects the endothelium from inflammation and a pro-thrombotic state

Our further studies uncovered a beneficial role of Sirt6 in arterial thrombosis and suggest that Sirt6 delays thrombosis by protecting the endothelium. It would be interesting to assess if Sirt6 can do this more efficiently when it is activated or overexpressed in the endothelium.

While Sirt6-dependent retention of NF-κB and AP-1 signalling is in the centre of attention, the exact underlying mechanism remains unclear. Histone 3 acetylation levels should be assessed, along with functional studies of NF-κB and AP-1 DNA binding activity. ELISA-based activity assays, that can quantify the amounts of Sirt6-interaction partners RelA and c-JUN bound to DNA, are available. Alternatively, phosphorylation of c-JUN and the translocation of NF-κB to the nucleus could shed light on which, if only one of the transcription factors is playing a predominant role. Finally, the results obtained in cell cultures of HAECs, using a Sirt6 knockdown approach, should be verified in Sirt6$^{-/-}$ mouse endothelial cells.

7.5 Conclusions

For the first time, we describe a beneficial role for Sirt3 in major hallmarks of CVD, comprising risk factor development, endothelial dysfunction, and arterial thrombosis, and an advantageous role for endothelial Sirt6 in arterial thrombosis. Furthermore, our results emphasise the detrimental role of ROS and inflammatory signalling in these diseases.

Based on our results, we speculate that specific activation of Sirt3 and Sirt6 aids in the prevention and acute therapy of CVD. Currently, no sirtuin-specific activators are known. Investing in the discovery of new substances that can specifically activate certain sirtuins could open a whole new field of therapeutic possibilities for CVD, as well as other metabolic and age-related diseases.

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9 Curriculum Vitae

Name	GAUL
First names	Daniel Sebastian
Date of Birth	15 March 1986
Nationality	German

Education

2002-2005	**Friedrich Dessauer Gymnasium**, Frankfurt a. M., Germany Abitur 2005 (equivalent to Matura)
2006-2008	**University of Mainz**, Germany Pre-diploma examinations in Biology in 2008
2008-2009	**University of Glasgow**, Scotland, United Kingdom 3rd year full term honours course in Biomedical Sciences funded by an ERASMUS scholarship
2009-2012	**University of Mainz**, Germany Diploma in Genetics, Pharmacology & Toxicology, and Zoology in 2012 (equivalent to Master of Science)
2012	**Max Planck Institute for Heart and Lung Research** Bad Nauheim, Germany Diploma Thesis: 'Generation of a TEAD2 KO mouse line and identification of new potential interaction partners of the TEAD transcription factor family in smooth muscle cells'
2013-present	**University of Zurich**, Switzerland PhD student in the 'Integrative Molecular Medicine' programme at the 'Center for Molecular Cardiology'

Extracurricular Activities

2007-2012	**Founder member of the 'Biotechnological Students Initiative' (btS e.V.) Mainz** Board member 2009-2011; Chairman of the board 2011/12
2008-2009	**Member of the 'Glasgow University Student Biochemistry Society'**
2010	**Volunteer at San Cristobal Biological Reserve** Galapagos, Ecuador 6-week volunteer programme in reforestation, reserve maintenance, community activities and organic farming

2011	**Scientific Assistant** at the University of Mainz Supervision of practical undergraduate courses
2013-present	**Member of the 'Life Science Zurich Young Scientist Network'** Project Leader 2014/15; Board member 2015/16; Chairman of the Board 2016/17

Awards

2016	**Best Poster Award** at the 12th Symposium of the Zurich Center for Integrative Human Physiology in Zurich, Switzerland
2017	**Best Free Communication** at the Cardiovascular & Metabolic Research Meeting in Fribourg, Switzerland
2017	**Silver Poster Award** at the Cardiology Update Congress 2017 in Davos, Switzerland

Publications

Original Articles

2014	Winnik S, **Gaul DS**, Preitner F, Lohmann C, Weber J, Miranda MX, Liu Y, van Tits LJ, Mateos JM, Brokopp CE, Auwerx J, Thorens B, Lüscher TF, Matter CM. Deletion of Sirt3 does not affect atherosclerosis but accelerates weight gain and impairs rapid metabolic adaptation in LDL receptor knockout mice: implications for cardiovascular risk factor development. Basic Res Cardiol 2014;**109**(1):399.
2016	Winnik S, **Gaul DS**, Siciliani G, Lohmann C, Pasterk L, Calatayud N, Weber J, Eriksson U, Auwerx J, van Tits LJ, Lüscher TF, Matter CM. Mild endothelial dysfunction in Sirt3 knockout mice fed a high-cholesterol diet: protective role of a novel C/EBP-β-dependent feedback regulation of SOD2. Basic Res Cardiol 2016;**111**(3):33.
2017	Reiner MF, Akhmedov A, Stivala S, Keller S, **Gaul DS**, Bonetti NR, Savarese G, Glanzmann M, Zhu C, Ruf W, Yang Z, Matter CM, Lüscher TF, Camici GG, Beer JH. Ticagrelor, but not clopidogrel, reduces arterial thrombosis via endothelial tissue factor suppression. Cardiovasc Res 2017;**113**(1):61-69.

Reviews

2017	**Gaul DS**, Stein S, Matter CM. Neutrophils in cardiovascular disease. *Eur Heart J* 2017; **38**(22): 1702-4